The Early Role of the Transcription Factor COUP-TFI in Cortical Development.

Thesis

The Early Role of the Transcription Factor COUP-TFI in Cortical Development

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ABSTRACT

The mammalian neocortex is the seat of sensory and motor processing as well as higher brain functions. Its development is regulated by patterning centers and transcription factors expressed in temporally and spatially restricted fashions. Our understanding of the functions of such transcription factors during development has been limited by the pleiotropic effects and neonatal lethality resulting from null mutations. Chicken Ovalbumin Upstream Promoter Transcription Factor I (COUP-TFI) is a transcription factor expressed in the cortex during development, which has previously been implicated in cortical development by characterization of a *COUP-TFI* null mutant. In this report we described the use of a conditional mutant approach, in which *COUP-TFI* expression is eliminated exclusively from the cortex using lox-CRE technology, to investigate the role of COUP-TFI during cortical development. We have provided preliminary evidence for a novel role of COUP-TFI as a pro-differentiation factor in early cortical neurogenesis and as a regulator of neuronal diversity during the formation of the cortical plate. COUP-TFI appears to act as a regulator of upper versus lower layer neuronal cell fate in a way that is reminiscent of the function of its fly homolog, sevenup, in regulating early versus late neuronal fate in the fly nervous system. The elimination of COUP-TFI from the cortex also allowed us to identify its role outside of the cortex in guiding thalamocortical axons through the internal capsule. These data provide exciting prospects prospects for further insights into transcriptional programs regulating cortical development.

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I. INTRODUCTION

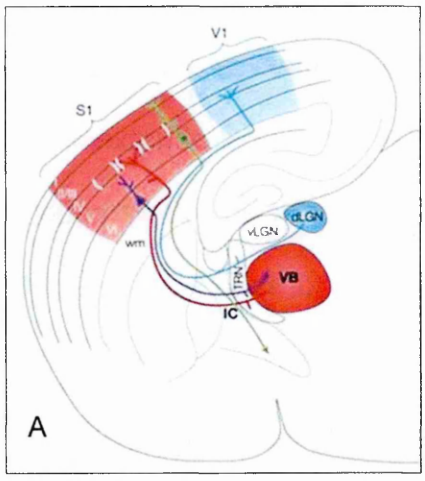
1. Cortical Development: Cortical Neurogenesis and Radial Migration

The mammalian forebrain is characterised by the presence of the neocortex, the seat of sensory and motor processing as well as higher conscious and subconscious brain functions. The neocortex consists of six histologically distinct layers in its radial dimension, in which each layer consists of neuronal cell bodies and projections to and from different cortical and subcortical areas (Fig. 1a, reviewed in Rash and Grove, 2006).

During development, the cerebral cortex forms from the anterior neural tube, and the mitotic progenitors that give rise to the cortical plate are located in the ventricular zone. Neurons born in the ventricular zone migrate radially to the surface of the neural tube to form a layer of early-born neurons called the preplate. During mouse development, this happens around 10.5 days after fertilization (embryonic day 10.5 or E10.5). The preplate is a transient structure and is then split into marginal zone and subplate by later born neurons migrating from the ventricular zone and settling between the two layers, forming the cortical plate (Fig. 1b). The preplate, marginal zone and subplate neurons have key functions during cortical histogenesis, but in rodent a large proportion of both populations are lost in the adult once the cortex is fully formed (Ferrer et al., 1990).

The neurons that settle in layers between the subplate and marginal zone are generated in a precise temporal order. The generation of these layers was extensively investigated using H^3-thymidine labelling studies, which have shown that the birth of the layers of the cortical plate follows an inside-out order (Rakic, 1974; McConnell et al., 1988). Early born neurons give rise to the deepest layers,

while later born neurons give rise to the superficial layers, with the exception of layer I. During mouse development, layer VI neurons are born around E13.5-15.5, followed by birth of layer V (E14.5- 16.5), IV and II/III (E16.5-19.5). Cortical development is generally completed by the first week after birth.



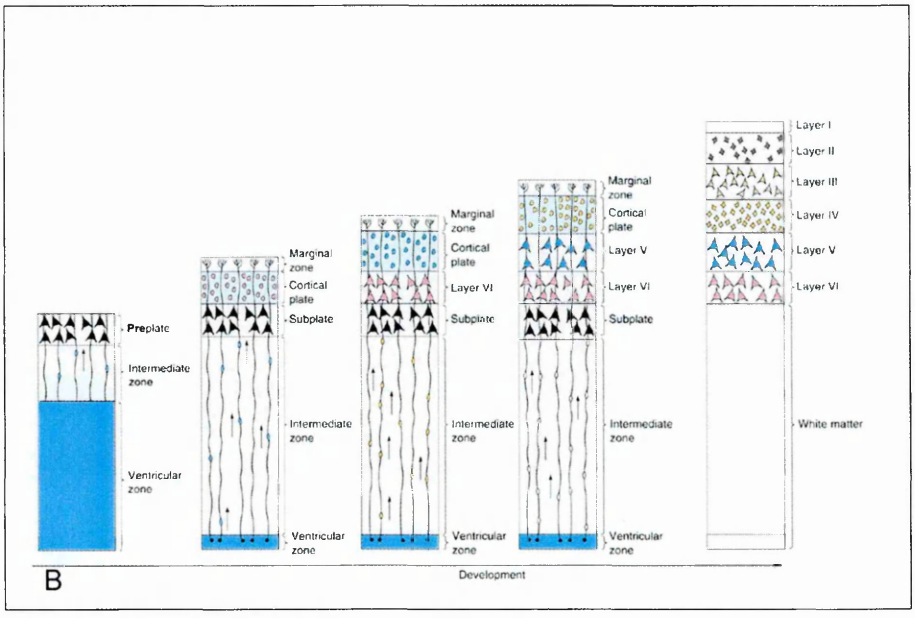


Fig. 1. (a). Coronal forebrain hemisection, showing cortical layers I-VI and layer-specific projection patterns. The red line indicates thalamocortical axons running from the ventrobasal complex of the thalamus (VB) to the primary somatosensory cortex (S1) layer IV. Neurons in layer VI of the same area project to the thalamus. IC, internal capsule; S1, primary somatosensory cortex; TRN, thalamic reticular nucleus; vLGN, ventral lateral geniculate nucleus; WM, white matter. Taken from Lopez-Bendito and Molnar, 2003. (b). A schematic representation of cortical neurogenesis and lamination. Neurons are born in a temporal sequence with early-born neurons forming deeper layers (VI, V) and late-born neurons migrating past early-born neurons to form upper layers.

The progenitors that give rise to cortical neurons are located in the ventricular and subventricular zones and give rise to neurons either by symmetric or asymmetric divisions (Angevine and Sidman, 1961; Luskin, 1993; reviewed in Fishell and Kriegstein, 2003). A symmetric division is one in which mitosis gives rise to two morphologically identical neurons, which proceed to behave in the same way in terms of migration and division. These cells could be either two neurons or two progenitors that will continue to divide. Asymmetric divisions are characterized by the birth of two morphologically distinct cells, such as one neuron and one progenitor cell. The incidence and location of symmetric and asymmetric divisions change throughout corticogenesis. Before neurons start to differentiate, the telencephalon consists exclusively of neuroepithelial cells, which undergo symmetric divisions to enlarge the progenitor pool. Then, during early corticogenesis, from E10-E13, the germinal layer is the site of both symmetric divisions to enlarge the progenitor pool, as well as asymmetric divisions, which give rise to one neuron and one progenitor. The neurons migrate radially outwards, while the progenitor will remain in the germinal layer. Progenitors that have already undergone one division may migrate to the basal layer of the ventricular zone to form the subventricular zone, where they may continue to divide to produce two neurons. These cells are called intermediate progenitors (Haubensak et al., 2004; Miyata et al., 2004; Englund et al., 2005).

As neurogenesis progresses, the number of intermediate precursors increases, and most neurons are born from asymmetric or symmetric divisions of radial glial cells in the subventricular zone (Haubensak et al., 2004, Englund et al., 2005). Asymmetric, neuron-producing divisions may also take place in the ventricular zone. During late stages of neurogenesis, E16-E19, the proportion of symmetric

divisions increases again, but this time intermediate progenitors divide to produce two neurons, which migrate to the upper layers (Haydar et al., 2003) The process of migration has been divided into four phases, which consist of an initial phase in which neurons assume a bipolar shape and if in the ventricular zone, migrate to the subventricular zone. Here, during the second phase, they stall in the subventricular zone for about one day. This is followed by a third phase in which a process is extended towards the ventricle and the neurons undergoes somal translocation back towards the ventricle. Once the process has contacted the ventricle, the final phase of radial migration outwards begins. During this stage, the neurons reverses its polarity, such that the process is orientated outwards towards the cortical plate, and begins migrating along a radial glial process towards its target layer (Noctor et al., 2004). The target layer is determined by the neuronal birthdate as described above, and the stopping point of migration is regulated by the presence of Reelin in the marginal zone (reviewd by Tissir and Goffinet, 2003). After radial glial cells have given rise to neurons, they may continue to divide to give rise to cells with a glial fate.

While the temporal aspect of neurogenesis has been well described, it remains unclear how the passage of time and the passage through a program of symmetric and asymmetric divisions in the proliferative zones is linked to molecular changes in those zones over time. Genetic and pharmacological manipulations have been described that can give rise to perturbations in the passage through such a program (Calegari and Huttner, 2003; Yun et al., 2004). Many of these manipulations interfere with the cell cycle or mitosis. For example, using cultured neuroepithelial cells, the application of olomoucine, which specifically inhibits cyclin-dependent kinases and G1 progression, is able to

lengthen the cell cycle, resulting in the premature expression of a marker of intermediate progenitors (*Tis21*) and the overproduction of neurons, depleting the progenitors (Calegari and Huttner, 2003). An *in vivo* use of the drug could hence be imagined to result in an overproduction of early -born and underproduction of late-born neurons. Along these lines it has been seen that the mutation of a gene, *Id4*, which is required for G1-S transition, results in decreased proliferation of ventricular zone stem cells and their premature differentiation (Yun et al., 2004). This means that at early stages of corticogenesis, until E15.5, the Id4 mutant has a thicker cortical plate, but during later stages, the depletion of the progenitor pool has the consequence that the cortical plate becomes increasingly thinner with respect to wild-type, resulting in an abnormally small brain (Yun et al., 2004). A similar phenotype has been described in mice carrying a mutation in *Nde1*, a gene required for centrosome duplication and mitotic spindle assembly and in mice with a mutation in α-*SNAP*, a synaptic protein which is required for apical protein localization and regulation of symmetry of division during cortical development (Chae et al., 2004).

These experiments raised the question of whether the relationship between neuronal birthdate and laminar fate was influenced by cell-autonomous or cell non-autonomous factors. Early transplantation experiments that were instructive about the relationship between neuronal birthdate and laminar fate indicated that fate was determined primarily by extrinsic cues. Labelling with ^{3}H-thymidine and transplantation of progenitors giving rise to layers V and VI into a host that was in a later stage of corticogenesis, in which progenitors were giving rise to layers II and III, resulted in neurons migrating to the host-appropriate layer if transplanted before their terminal division (McConnell and Kaznowski, 1991). This flexibility in

fate indicates that early progenitors have the potential to give rise to neurons that migrate to the host layer, suggesting that progenitors can respond to extrinsic cues in the ventricular zone. However, as the progenitors age their potential appears to become restricted. In the reverse experiment, in which progenitors giving rise to layers II and III were transplanted into a host brain that was in an earlier developmental stage (in which progenitors were giving rise to layers V and VI), the donor cells were not able to adopt a host fate, but waited in the ventricular zone until the host progenitors were forming layers II and III (Franz and McConnell, 1996). Finally, the transplantation of progenitors from mid-stage of neurogenesis, during which layer IV is being born, into an older environment allowed them to adopt the host fate, while transplantation into a younger environment resulted in neurons migrating to the donor appropriate layer, or to layer V, which was neither the donor nor host appropriate layer (Desai and McConnell, 2000). These experiments show that fate determination takes place during or before the final progenitor division, the restriction of fate is prolonged with respect to the production of a cortical layer, and that fate is determined by both intrinsic and extrinsic cues.

While these experiments indicated intrinsic determinants exist, it is only more recently that such genes have been identified. For a gene to be considered an intrinsic determinant, one would expect loss-of-function to result in a misspecification of laminar fate. The gene should be expressed in the ventricular zone as the layer is being generated, as fate determination has been found to be determined before or during the terminal division, and the gene might continue to be expressed in the layer. Furthermore, such genes are likely to be transcription factors that control other regulators of neuronal differentiation, such as

neurotransmitters, guidance receptors and/or guidance cues. In fly, a cascade of transcription factors that act as intrinsic markers of neuronal temporal identity has been described (Isshiki et al., 2001; Grosskortenhaus et al., 2005), and it is possible to imagine that laminar identity in mouse cortex may partially be controlled by similar mechanisms. Recently, *in vitro* experiments have shown that cortical progenitors in culture give rise to neurons expressing layer-specific markers in the same order and timing that is seen *in vivo*, suggesting that the developmental program required to give rise to neuronal fates of different layers is intrinsic to individual progenitors (Shen et al., 2006).

So far, three genes have been identified that fulfil these conditions. Francois Guillemot and colleagues have identified the *Neurogenin-1* (Ngn1) and *2* (Ngn2) genes as regulators of cortical glutamatergic fate of early born, lower-layer neurons (Schuurmans et al., 2004). *Neurogenins* are both expressed exclusively in progenitors, and mutants for either *neurogenin* individually suggest that there is some redundancy between the two genes. Indeed, *Ngn1,2* double mutants display a decrease or disorganization of lower-layer markers, while upper layer markers appeared intact. Furthermore, corticofugal projections, which arise from layers V and VI, were disturbed. These results suggest that the generation of lower cortical layers is neurogenin-dependent, while upper layers can form normally in the absence of neurogenins.

Foxg1 is the third gene which has been identified as an intrinsic determinant of laminar fate. *Foxg1* is a member of winged-helix forkhead box family of transcription factors (Xuan et al., 1995). It is expressed in the ventricular zone once the preplate and subplate have formed, and continues to be expressed throughout the generation of layers II-VI. In the absence of Foxg1, the cortical

plate is not formed, but instead layer I neurons expressing Reelin are overproduced. This suggests that Foxg1 is a suppressor of layer I, Reelin-positive, neuronal identity and a determinant of later cell fates (Hanashima et al., 2004). Hence, as our knowledge of the molecular aspects of cortical development increases, we are finding genetic correlates of temporal and laminar identity that will eventually contribute to a molecular description of cortical development in the radial dimension. It is likely that many other genes act as molecular determinants of temporal identity as well as regulators of switching between temporal identities (reviewed in Hevner et al., 2005).

2. Cortical Arealization is Regulated by Morphogens and Transcription Factors

In the tangential dimension, our understanding of the molecular characterization of cortical areas is more advanced. Research into the mechanisms underlying cortical arealization has been driven by two principal hypotheses. The first hypothesis proposed that area identity is imparted to neurons at birth in the ventricular zone, and that this area identity is maintained when they migrate to the appropriate layer. Hence the ventricular zone was thought to intrinsically contain information that mapped the cortical areas, and this hypothesis is called the protomap model (Rakic et al., 1991). A competing hypothesis was put forth by the protocortex model (reviewed in O'Leary et al., 1992), which proposed that cortical tissue is equivalent across the cortex when generated and that cortical area identity is extrinsically determined by thalamocortical (TC) innervation. This hypothesis was supported by evidence from heterotopic transplantation experiments. In rodents, in which the sense of touch by whiskers is highly developed, axons from the ventrobasal thalamic nucleus innervate layer IV of the

future somatosensory cortex. Layer IV neurons then aggregate around the axon terminals to form barrels that represent the whiskers (van der Loos and Woolsey, 1973). If cortical tissue is transplanted from late embryonic visual cortex into neonatal somatosensory cortex, the donor tissue of visual cortex origin is innervated by ventrobasal axons and the thalamic innervation directs the formation of barrels (Schlaggar and O'Leary, 1991).

While it is intuitive that thalamocortical innervation is crucial in attributing a functional identity to all cortical areas, this does not imply that cortical areas are uncharacterised prior to thalamic innervation. More recently, evidence has accrued suggesting that cortical areas are not equivalent prior to thalamic innervation. A number of genes, such as *RORβ*, *Id2* and *Cadherin8*, are expressed in an area-specific manner within the cortical plate, and these area-specific patterns of gene expression may be the result of intrinsic genetic mechanisms in the ventricular zone imparting area identity early on during development. Strong evidence for the protomap model came from the *Gbx2* null mouse (Miyashita-Lin et al., 1999). *Gbx2* is a transcription factor expressed specifically in the dorsal thalamus, but not in the cortex (Miyashita-Lin et al., 1999). The Rubenstein laboratory showed that in the absence of Gbx2, expression of thalamic markers such as Id4 is disrupted and the growth of thalamic axons is impaired. DiI labelling showed that thalamocortical axons did not innervate the subplate, but that the expression of cortical area- and lamina-specific markers *Id2*, *EphA7* and *RORβ* did not show any obvious difference from wild-type in the *Gbx2* mutant at P0 (Miyashita-Lin et al., 1999). Similarly, mice mutant for *Mash-1,* another transcription factor expressed in the thalamus but not cortex, also fail to develop thalamocortical projections, but show wild-type

expression of cortical region- and layer-specific late-expressing markers such as *Lhx2*, *SCIP*, *Emx2* and *Cad8* (Nakagawa et al., 1999).

Since these mutant mice have established that there are mechanisms intrinsic to the cortex that act during arealization, a number of morphogens and transcription factors that contribute to the process have been identified (see Fig. 2 below). For example, the anterior pole of the dorsal telencephalon expresses members of the fibroblast growth factor family, such as *Fgf7, 8, 11* and *13* (Yaylaoglu et al., 2005), while the cortical hem, situated most medially in the developing cortex where the two hemispheres meet, expresses multiple *Wnt* and *Bmp* genes (Grove et al., 1998; Shimogori et al., 2004). Manipulations of the anterior source of *Fgf8* have shown that the levels and location of FGF8 secretion affects the size and position of cortical areas, such as somatosensory cortex (Fukuchi-Shimogori and Grove, 2001). Furthermore, WNT and BMP molecules produced at the cortical hem and FGF8 produced at the anterior pole can regulate each other.

A number of transcription factors that are regulated by these morphogens have been identified, and include *Emx2*, *Pax6* and *COUP-TFI*. These genes are expressed in gradients within the ventricular zone, making them good candidates for imparting area-specific character to developing cortical tissue (see Fig. 2 below). Mutant mice lacking theses genes have been described as displaying shifts in area-specific markers, in barrel location and in topographic projections from thalamus to cortex (Bishop et al., 2000; Zhou et al., 1999, 2001). In the wild-type mouse, *Emx2* is expressed in a high caudomedial to low rostrolateral gradient, while Pax6 is expressed in a high rostrolateral to low caudomedial gradient and *COUP-TFI* is expressed in a high caudolateral to low rostromedial gradient. The loss of *Emx2* resulted in an expansion of rostrolateral areas, while

caudomedial areas were reduced (Bishop et al., 2000). Thalamocortical projections from the ventrobasal nucleus of the thalamus were shifted posteriorly, which is consistent with a caudal shift of somatosensory cortex. In the naturally occurring small eyes mutant, that lacks *Pax6*, the opposite phenotype was reported such that markers like RORβ, Id2 and p75 were indicative of an expansion of caudal cortical identity (Bishop et al., 2000, 2002). These markers were also disrupted in the *COUP-TFI* null mutant as is described in more detail below.

While we now have ample evidence that genes exist that intrinsically pattern the cortex, it is important to consider how these genes might regulate what characterizes an "area". For example, caudal pallium, which is where somatosensory (parietal cortex) and visual areas (occipital cortex) develop is characterized by a thick layer IV, which received sensory inputs from thalamic axons, and thick upper layers, which give rise to cortico-cortical projections. At the same time, somatosensory cortex displays thinner layers V and VI (which give rise to corticothalamic projections), with respect to the more rostral motor cortex (reviewed in Shipp, 2005) Hence molecules suchas Emx2 and Pax6 are likely to regulate the genesis of the correct number of neurons in each layer at a particular rostro-caudal position and must guide thalamic axons to the correct target within the cortex. In considering the function of these gradients of gene expression, it is worthwhile considering that the neurogenetic gradient of the cortex develops from rostral to caudal, and from ventral to dorsal (Bayer and Altman, 1987). Within the neurogenetic gradients, local accelerations of cell divisions arise that result in functionally relevant area-specific differences in the thicknesses of layers (Polleux et al., 1997).

It is likely that the gradients of expression of genes such as *Emx2*, *Pax6* and *COUP-TFI* are instrumental in setting up reported neurogenetic gradients and regional differences in cell cycle rates. It has been reported that a loss of Pax6 has an increased proliferative effect and produces defects in differentiation both *in vitro* and *in vivo* (Hack et al., 2004; Warren et al., 1999), and that COUP-TFI regulates neuronal and oligodendrocyte differentiation (Studer et al., 2005; Adam et al., 2000; Yamaguchi et al., 2004). Given that these and many other transcription factors that regulate proliferation and differentiation are expressed in gradients, it is possible to imagine how they might mediate graded neurogenesis across the neocortex and how the combination of many gradients can lead to temporal and regional differences in proliferative dynamics.

3. Interactions of Cortical Patterning and Thalamocortical Projection

Beyond the intrinsic factors regulating cortical development and arealization described above, thalamocortical axons are the principal extrinsic component that has been suggested to contribute to cortical arealization (Schlaggar and O'Leary, 1991). Thalamocortical axons grow ventrally from the dorsal thalamus and turn dorsolaterally towards the diencephalic-telencephalic boundary to enter the internal capsule at E13. They then grow toward perireticular cells of the internal capsule where they stall until E15 before crossing the pallial-subpallial boundary (Molnar et al., 1998). On the other side of this boundary, corticofugal axons pause before both corticofugal and thalamocortical projections proceed to grow towards their targets, interacting closely with each other as they grow. While transplantation studies such as those described by O'Leary above suggested that thalamocortical axons are a potent organizer of cortical area identity, the *Gbx2*

and *Mash1* mutant mice suggest that region-specific gene expression is established before the arrival and in the absence of thalamocortical projections.

Given the evidence that thalamocortical axons are not the primary factor driving cortical arealization, the opposite hypothesis has been investigated: To what extent are the molecules that pattern the cortex responsible for the formation of an intact thalamocortical projection? The pathfinding defects seen in mutants for molecules expressed in an area-specific manner in the cortex, such as Ephrins, as well as the mutants of upstream transcription factors such as *COUP-TFI* and *Emx2*, have been used to suggest that thalamocortical axons are guided to their cortical targets by the intrinsic cortical patterning (Dufour et al., 2003, Bishop et al., 2000; Zhou et al., 1999). Defects in thalamocortical pathfinding seen in these mutants could result directly from a requirement for a correctly patterned cortex to guide thalamocortical axons to their targets, or from reciprocal interaction between thalamocortical and corticothalamic axons during their outgrowth, which has been described as the "handshake hypothesis" (Hevner et al., 2002).

More recently, the importance of intermediate targets of thalamocortical axons in pathfinding to the cortex has been described. The internal capsule in the ventral telencephalon is the site of stalling of thalamocortical axons before growing towards the cortex and interacting with corticofugal axons (reviewed in Lopez-Bendito and Molnar, 2003). Evidence for the importance of the ventral telencephalon came from the *Ebf1* and *Dlx1/2* mutants, which do not display defects in cortical or in thalamic patterning, but due to the loss of their expression in areas surrounding the internal capsule, result in a defect in the topography of thalamocortical axons in the basal ganglia, and this topographical defect is propagated as the axons project to the cortex (Garel and Rubenstein, 2002). Furthermore, reciprocal gradients of ephrin-A5 ligand

in cortex and Eph-A4 receptor in thalamus have been described to be required for the formation of an intact topographical projection of thamocortical axons within a cortical area, and that an independent ephrin-A5 ligand gradient in the ventral telencephalon is required for the formation of a correct topographical organization of axons between cortical areas (Dufour et al., 2003). It has also been reported that the permissive area through which axons migrate in the internal capsule is characterized by Neuregulin expression in cells that have migrated from the lateral ganglionic eminence to the internal capsule (Lopez-Bendito et al., 2006). The contribution of ventrally expressed transcription factors and guidance cues, such as ephrin-A5, Neuregulins, and Ebf1, to thalamocortical pathfinding, also suggest that caution should be used when interpreting the thalamocortical phenotypes of null mutants of genes expressed in more than one area along the path from thalamus to cortex, as is the case with *COUP-TFI*. The figure below illustrates the contributions of cortical patterning centers described above and of projections between thalamus and cortex in generating cortical areas (Fig. 2).

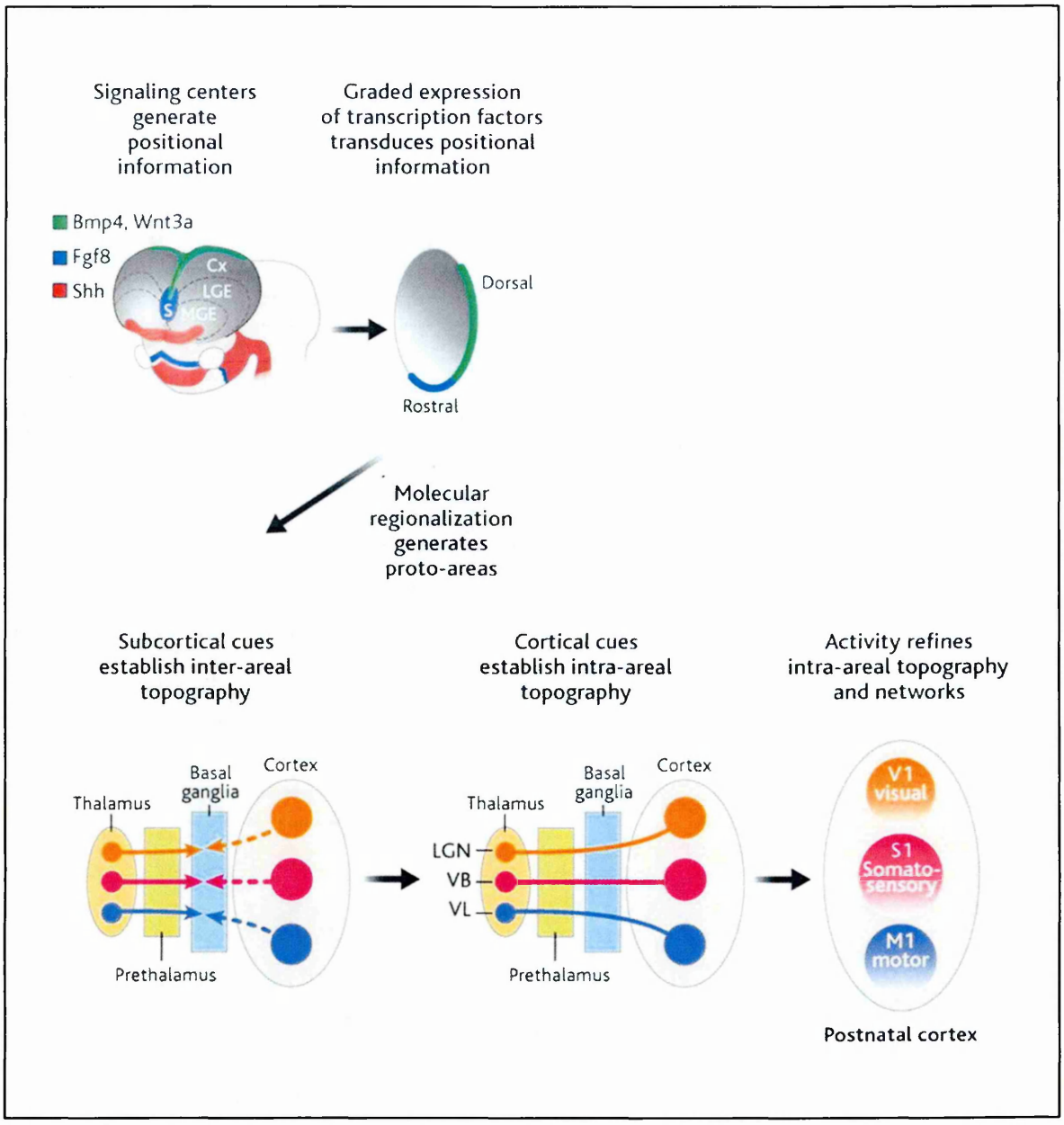


Fig. 2. Steps in generating cortical areas: Secreted proteins, such as FGF8 and Wnt3a, from patterning centers generate positional information. These in turn regulate gradients of transcription factor (such as Emx2, Pax6) expression in the cortical ventricular zone, conferring regional identities to its derivative, the cortical plate, forming proto-areas. The topography of axons growing from the cortical plate and thalamus is regulated by intermediate targets (e.g. internal capsule). This generates a coarse inter-areal projection map, which is refined by activity-dependent mechanisms (not discussed here). Cx, cortex; LGE, lateral ganglionic eminence (striatum); LGN, lateral geniculate nucleus; MGE, medial ganglionic eminence (pallidum); S, septum; VB, ventrobasal thalamus; VL, ventrolateral thalamus. Adapted from Sur and Rubenstein, 2005.

4. *COUP-TFI*: a nuclear receptor of the steroid/thyroid receptor superfamily

COUP-TFI (Chicken Ovalbumin Upstream Transcription Factor I) is an orphan receptor of the steroid/thyroid receptor superfamily. COUP-TFI, like other nuclear hormone receptors, is a transcription factor that shows high structural and functional conservation with the other members of the same family and is characterized by three signature domains, a ligand binding domain, a DNA binding domain and a transactivation domain (Wang et al., 1989; Fig. 3). This family includes receptors for steroid, thyroid and retinoid hormones, some of which do not have molecularly identified ligands and are hence termed orphan receptors, such as COUP-TFI. COUP-TFI was initially identified in chicken as an activator of the ovalbumin gene (Sagami et al., 1986), after which both homologues were identified in a number of species including humans and mouse (Wang et al., 1989; Qiu et al., 1994). COUP-TFI has a homolog, COUP-TFII with which it shares sequence homology and an overlapping expression pattern in some tissues, but is functionally distinct.

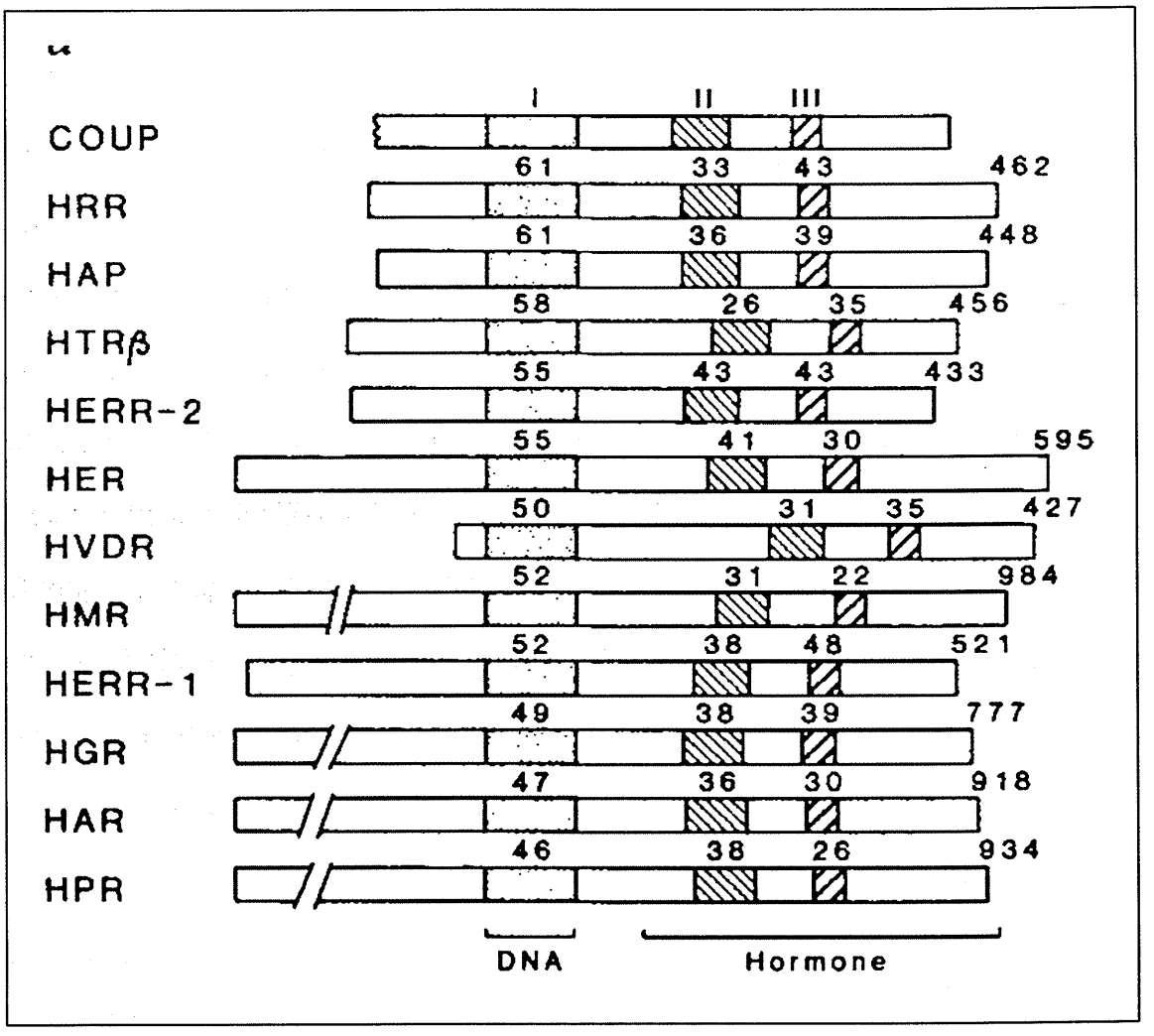


Fig. 3. COUP-TFI is a member of the steroid-hormone receptor superfamily. Regions I (66 amino acids), II (42 aa) and III (23 aa) define regions of similarity with other human steroid-hormone receptors. I represents the zinc-finger DNA binding domain, II and III are stretches within the ligand binding domain. The number above each box represents the percentage of similarity in each region with respect to the corresponding region in COUP-TFI. HRR: human retinoic acid receptor; HAP (retinoic acid receptor b); HTRß: human thyroid receptor beta; HERR: human estrogen receptor related 1 or 2; HER: human estrogen receptor; HVDR: human vitamin D receptor; HMR: human mineralocorticoid receptor; HGR: human glucocorticoid receptor; HAR: human androgen receptor; HPR: human progesterone receptor. Taken from Wang et al., 1989.

While COUP-TFI was initially identified as a transcriptional activator, the majority of studies that have followed have suggested a role for COUP-TFI as a repressor of

genes regulated by other nuclear hormone receptors. These include genes that are transcriptionally activated by the retinoic acid receptor, thyroid hormone receptor, vitamin D receptor, and hepatocyte nuclear factor 4. COUP-TFI has also been found to act together with other nuclear hormone receptors via heterodimerization, or to dimerize with itself (Kyakumoto et al., 1999; Cooney et al., 1992, Mietus-Snyder et al., 1992). Furthermore, COUP-TFI has been found to interact with the estrogen receptor, bind the estrogen response element and either repress genes regulated by the estrogen receptor, or activate them depending on the sequences flanking the estrogen response element (Klinge et al., 1997, 1999). It also represses a glutamate receptor subunit in monkey epithelial CV1 and rat CG4 oligodendrocytes (Chew et al., 1999). Transcription mediated by COUP-TFI has been reported to be regulated by CaMKIV (Kane et al., 2000). Interestingly, many of the genes that have been identified as targets of positive regulation of COUP-TFI are involved in neuronal identity. In general, COUP-TFI has been proposed to repress transcription via competition for DNA binding sites, competition for dimerization with Retinoid X receptor, active suppression by a putative silencing domain in the ligand binding domain, or via trans-repression by binding the ligand binding domains of other nuclear hormone receptors (reviewed in Park et al., 2003).

The targets identified to date delineate COUP-TFI as an effector of events regulated by retinoic acid, as well as a regulator of cellular differentiation, which puts it in a good position to integrate these two events, such as is required during nervous system development. *In vitro*, overexpression of *COUP-TFI* has been found to antagonize retinoic acid induced neuronal differentiation of teratocarcinoma PCC7 cells (Neuman et al., 1995). COUP-TFI was also found to antagonize retinoic-acid induced differentiation in P19 embryonic carcinoma cells at high expression levels, but to

promote differentiation at low expression levels, while a dominant negative COUP-TFI construct resulted in apoptosis (Adam et al., 2000). In the same set of experiments, dominant negative COUP-TFI inhibited neurite outgrowth, while overexpression facilitated neuronal migration (Adam et al., 2000). A role for COUP-TFI in regulating neuronal migration has been supported in the brain in organotypic slices, where the rate of migration was seen to increase upon overexpression of *COUP-TFI* (Tripodi et al., 2004). In the same system COUP-TFI has also been described as a repressor of Cajal Retzius cell differentiation (Studer et al., 2005).

During development *in vivo*, *COUP-TFI* has been functionally implicated in a wide range of biological processes in the adult and during development, including bone morphogenesis, inner ear development, cardiac development and development of the central nervous system (reviewed in Park et al., 2003). Furthermore, a role for COUP-TFI in oligodendrocytic differentiation has been described. The *COUP-TFI* mutant mouse has delayed myelination, delayed differentiation of oligodendrocytes and deregulates SCIP, a POU domain transcription factor involved in axon myelination (Yamaguchi et al., 2004). The loss of *COUP-TFI* has been reported to produce defects in the development of the glossopharyngeal ganglion, its axonal outgrowth and arborisation as well as axon guidance and arealization in cortical development, which I will describe in more detail below (Qiu et al., 1997; Zhou et al., 1999, 2001).

Some regulatory mechanisms of COUP-TFI at the transcriptional level have been identified. These include regulation by Ets-1 and other Ets-factors (Salas et al., 2002), negative regulation by TGF-β signalling, and decreased expression in tumour tissue of breast cancer, esophegal cancer and ACTH-secreting tumor cells (Calonge et al., 2004; Chen et al., 2001; Xu et al., 2005; Paez-Pereda et al., 2001). *In vivo*, in early somite embryos COUP-TFI expression has been found to be upregulated by all-trans

retinoic acid, and in cortex of Fgf8 hypomorphs, COUP-TFI expression is rostrally expanded (Clotman et al., 1998; Storm et al., 2006).. Finally, at the protein level, COUP-TFI activity has been reported to be regulated by phosphorylation by protein kinase C and mitogen-activated protein kinase signalling pathways (Gay et al., 2002).

5. The Role of COUP-TFI in Cortical Development

A timecourse of COUP-TFI expression in the cortex was first described by Tsai and colleagues who reported that expression could be detected at E10.5 in caudal and ventral regions of the pallium (Qiu et al., 1994). At E13.5 expression was detected in the entire pallium, while at E18.5 and later, expression had decreased to background levels (Qiu et al., 1994). A functional role for COUP-TFI in cortical development was not suggested until the COUP-TFI knockout mouse was described (Zhou et al., 1999).

The principal findings of the paper describing the *COUP-TFI* knockout were that *COUP-TFI* null mutants displayed a progressive loss of layer IV cells, showed a thalamocortical pathfinding defect as well as a mis-differentiation and a progressive loss of subplate cells. Reduction of layer IV cells was shown by Nissl staining and *in situ* hybridization for a molecular marker of layer IV, RORβ. Immunohistochemistry against BrdU injected while layer IV cells are generated (E15-E15.5) appeared normal, suggesting that proliferation of putative layer IV was,not affected. BrdU labelling studies at E13.5, E14.5 and E15.5 were carried out to show that birth and radial migration of neurons in layers other than layer IV were intact. Hence Tsai and colleagues concluded that the loss of layer IV was due to progressive cell death. As thalamocortical innervation had previously been associated with layer IV survival (Windrem and Finlay, 1991), the possibility of a thalamocortical axon defect was

considered. DiI staining revealed indeed a defect in thalamocortical axon guidance around the internal capsule. Tsai and colleagues showed also that the *COUP-TFI* mutant displayed defects in subplate development. Subplate neurons are the initial cortical target of thalamocortical axons, which they reach around E15. Before innervating the cortex, thalamocortical axons stall at the subplate for a 2-3 day waiting period, during which they form contacts with subplate neurons. Defects in the development of the initial target of thalamocortical axons could underlie the pathfinding defect. In the null mutant until E16.5, staining with the subplate markers CSPG and NGFR p75 displayed normal subplate development, however the absence of calretinin at all stages in mutant tissue suggested that the subplate might undergo compromised differentiation. Nissl staining at P0, and immunostaining at P0 against BrdU injected at E11.5 showed a late loss of subplate neurons. The authors therefore propose that subplate neurons, which might secrete guidance cues required for thalamocortical axons to reach their target, do not differentiate correctly and are progressively lost.

On the basis of these results, the authors concluded that in the absence of COUP-TFI, subplate neurons differentiated incorrectly, leading to defective thalamocortical axon pathfinding. The fact that thalamocortical axons did not arrive in the cortex was then used to explain the death of subplate neurons and layer IV, as thalamic axons might provide trophic support or electrical activity necessary for the survival of these neurons. However, the physical distance between the internal capsule and subplate is most likely too large for secreted axon guidance molecules to act. Furthermore, the gradual loss of subplate cells cannot fully explain the thalamocortical axonal defect, since at the stage when these projections reach the cortex, subplate cells seem to be

present (as seen by CSPG and p75). This suggests that COUP-TFI may have independent developmental roles in both cortex and thalamus.

The O'Leary laboratory has provided an in-depth description of the distribution of COUP-TFI mRNA in developing rat neocortex (Liu et al., 2000). *COUP-TFI* was identified as a gene expressed in an area-specific manner before the cortex is innervated by thalamocortical axons in a screen using differential display PCR. A series of *in situ* hybridizations at different developmental stages revealed that COUP-TFI is expressed in a high caudal to low rostral gradient. At E14 to E16 of rat development, expression is seen in the ventricular zone and from E19 onwards, expression is maintained in the ventricular zone and turned on in the cortical plate. Once layers have differentiated, COUP-TFI is expressed in both a region specific (high caudal and lateral, low rostral and medial) and layer-specific manner. Expression is reported to be high in upper layers IV and II/III, and low in lower layers V and VI. Furthermore, COUP-TFI expression is described both in the thalamus and the internal capsule, leading the authors to suggest that axon pathfinding defects reported in the null mutant may instead due to the requirement for COUP-TFI in these areas, rather than due to subplate mis-differentiation. Similarly, high levels of COUP-TFI in layer IV suggest that layer IV cell death in the mutant may be cell autonomous rather than due to the absence of thalamocortical innervation.

Further evidence for distinct cortical and thalamic requirements for COUP-TFI has come from a description of shifts in regionalised markers in the COUP-TFI mutant before thalamocortical innervation (Zhou et al., 2001). The Tsai group reported disruption of the layer and region-specific expression of the markers RORβ, Id2, Cadherin and LAMP in the null mutant, while the markers Emx2 and Pax6 were reported to be intact. These defects were visible at E17.5, when thalamocortical

axons are stalled at the subplate, but have not yet massively innervated the cortical plate, suggesting that early cortical patterning might be intrinsic to the cortex. Using DiI tracing, Zhou and colleagues also showed that the corticothalamic axons arising from prospective visual cortex (caudal) of *COUP-TFI* null animals innervate the ventrobasal thalamus, rather than the lateral geniculate nucleus. This suggests that cortical axon pathfinding may be disrupted by changes in cortical area identity. The authors also reported that no changes in thalamic patterning markers were detected.

While our understanding of the roles of transcription factors in telencephalic development has increased in recent years with the widespread use of recombinant gene technologies in mice, many questions have arisen and have been left unanswered. While these studies in the null mutant irrevocably identify a role for COUP-TFI in cortical development, the fact is that a null mutation does not allow one to distinguish between requirements for COUP-TFI inside and outside the cortex. Furthermore, as the null mutants die at birth, they cannot act as a model in which to investigate the effects of *COUP-TFI* loss in the adult cortex when functional areas are well established. To overcome these experimental barriers in the null mutants, Maria Armentano in Dr. Michèle Studer's group has generated a *COUP-TFI* conditional mutant with the help of Cre-*Lox* technology. This conditional mutant can then be crossed with CRE-specific driver lines to investigate area-specific requirements for *COUP-TFI* in the mouse forebrain. This mutant line will be referred to as *COUP-TFI*^{flox}, while the null mutant line is referred to as *COUP-TFI*^{null}.

This study aims to describe the regionally restricted pattern of COUP-TFI expression in the *COUP-TFI*^{flox} mouse and to investigate the course of early cortical development in the absence of COUP-TFI in the cortex. Specifically, I will describe the use of region- and layer-specific molecular markers at different developmental stages to

outline a role for COUP-TFI in cortical neurogenesis and laminar differentiation, as well as the use of axonal tracers to characterize thalamocortical and corticothalamic axon pathfinding in the *COUP-TFI*^{flox} mutant.

II. RESULTS

1. Generation of the COUP-TFI conditional (*COUP-TFI^{flox}*) and null (*COUP-TFI^{null}*) mutant mouse lines and characterization of COUP-TFI expression in *COUP-TFI^{flox}* and *COUP-TFI^{null}* lines.

The *COUP-TFI^{null}* and *COUP-TFI^{flox}* mouse lines were generated by Maria Armentano in the lab of Dr. M. Studer by using the Cre-lox technology in embryonal stem (ES) cells and according to the schematic shown below (Fig.4). A gene targeting vector in which two lox sites flanking exon three containing the polyadenylation site of *COUP-TFI* and a third lox site downstream of the selectable neomycin (*neo*) resistance gene was correctly introduced into the endogenous *COUP-TFI* locus. To obtain *null* and *flox* alleles, the *COUP-TFI flox/neo* positive clone was electroporated with a plasmid coding for the *CRE*-recombinase. For the *null* allele, clones in which the third exon and the *neo* gene were excised were screened by PCR and identified *as COUP-TFI null* clones. For *flox* alleles, clones in which the *neo* gene only was excised were screened for and identified as COUP-TFI flox clones. These clones were subsequently injected into blastocysts and the resulting chimeras transmitted the modified alleles through the germline.

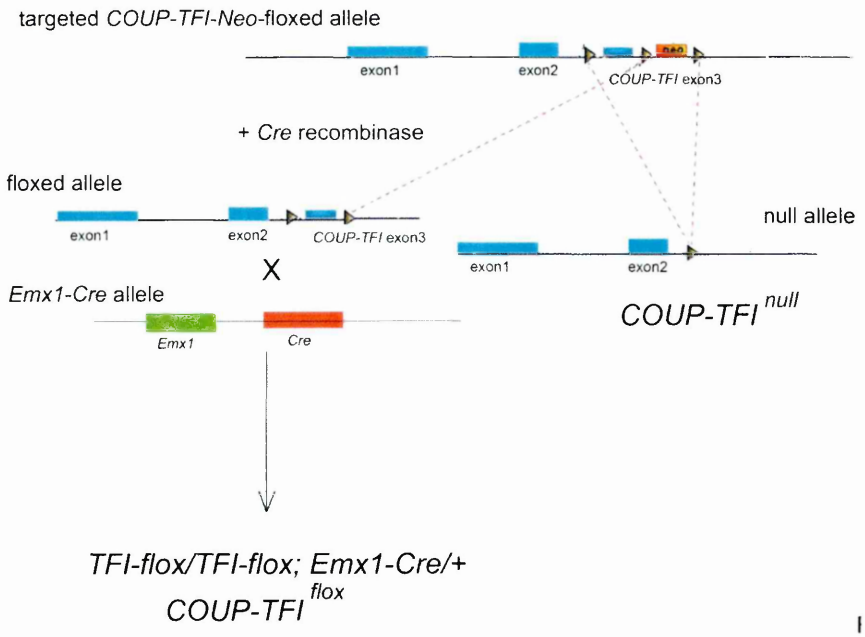
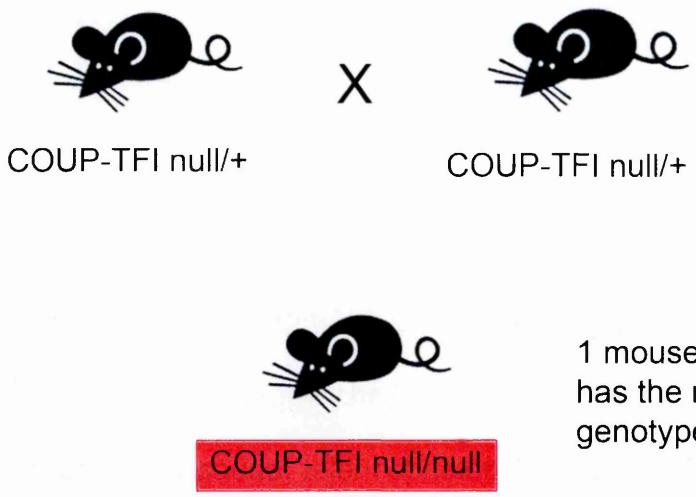


Fig.4. Schematic of gene targeting strategy: Cre-mediated recombination was implemented to generate a *null* allele and a *flox*ed allele from a gene targeting vector containing genomic *COUP-TFI* in which exon three is flanked by two *lox* sites and is upstream of a *neomycin* gene and a third *lox* site.

To obtain litters containing *COUP-TFI*^{null} mutant line mice, heterozygous *COUP-TFI*$^{null/+}$ male and female mice were mated and, as expected 25% of the progeny was homozygous for the null allele (Fig. 5). Pups died a few hours after birth as described before (Zhou et al., 1999).



Parental alleles	*COUP-TFI* wt	*COUP-TFI* null
COUP-TFI wt	+/+	+/-
COUP-TFI null	+/-	-/-

Fig. 5. Heterozygous null mice were mated with each other to produce *null/null* progeny at a frequency of 1 in 4.

COUP-TFI conditional mutants were generated by mating *COUP-TFI*^{flox} mice with the *Emx1-Cre* mouse line (kind gift of K. Jones) (Fig. 6). Selection of conditional mutants

was carried out using PCR amplification of *Cre* and *lox* loci, where both *COUP-TFI* alleles must be flanked by *lox* sites, and one *Cre* allele must be present (Fig. 6). The mouse line will be named *COUP-TFI*^{flox} throughout this work.

COUP-TFI lox/+;
Emx-1 Cre/+

X

COUP-TFI lox/lox
+/+

COUP- TFI lox/lox;
Emx-1 Cre/+

1 mouse in 4
has the required
genotype lox/lox;
Cre/+.

Parental Alleles	*COUP-TFI*^{lox}, *cre*	*COUP-TFI*^{lox} *wt*	*COUP-TFI*^{wt}, *cre*	*COUP-TFI*^{wt}, *wt*
COUP-TFI^{lox}, *wt*	*COUP-TFI*^{lox}/ *COUP-TFI*^{lox}, *cre/+*	*COUP-TFI*^{lox}/ *COUP-TFI*^{lox}, *+/+*	*COUP-TFI*^{wt}/ *COUP-TFI*^{wt} *cre/+*	*COUP-TFI*^{lox}/ *COUP-TFI*^{wt} *+/+*

Fig. 6. Mice carrying floxed *COUP-TFI* alleles and an *Emx-1 Cre* allele were mated to give rise to progeny with two floxed alleles and one *Emx-1-Cre* allele. In the example above, a *lox/+; Emx-1 Cre/+* animal was mated with a *lox/lox: +/+* animal to give rise to 1 in 4 animals that had both *lox/lox* and *Cre* alleles.

The expression of CRE under the *Emx1* promoter has previously been reported (Gorski et al., 2002), and the areas affected by a loss of COUP-TFI should be a reflection of the areas in which the CRE-recombinase is active. In the *Emx1-Cre* mouse, recombination was detected in the dorsal telencephalon at E10.5, while at E12.5 recombination was reported in most cells of the proliferative and postmitotic zones in the dorsal and ventral pallium as well as the cortical hem and choroid

plexus. To assess the depletion of COUP-TFI in the developing dorsal telencephalon, cortical sections of embryos at E10.5, 12.5, 13.5 and E15.5 were stained for COUP-TFI protein.

At E10.5, staining for COUP-TFI in the wild-type tissue shows expression throughout the germinal layer of the telencephalic hemispheres, Equivalent sections from *COUP-TFI*^{flox} tissue at this stage show a loss of protein in the ventral and dorsal pallium in both rostral and caudal telencephalon (Fig. 7. A', B', yellow arrowheads compare to Fig. 3A yellow arrow), but not in the subpallium, which maintains normal COUP-TFI expression levels as expected (Fig. 7., yellow asterisk). At this stage COUP-TFI has been almost fully inactivated, although a few positive cells are still detected in the pallium.

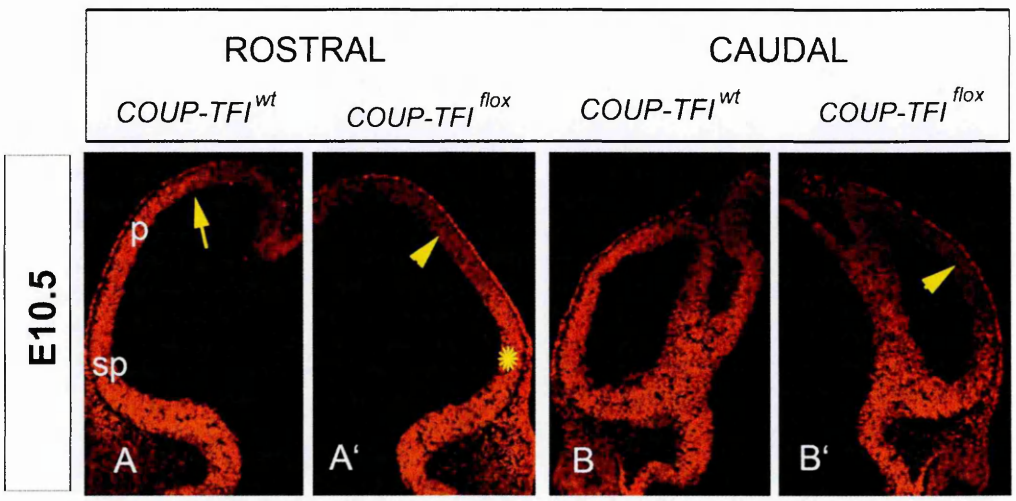


Fig. 7. Immunofluorescence of COUP-TFI expression in the telencephalon at E10.5. In wild-type sections expression can be seen in the pallium (arrow in A), while *COUP-TFI*^{flox} mutant shows a loss of COUP-TFI expression in the pallial region, but not in the subpallium (A': yellow arrowhead and asterisk). This loss of expression of COUP-TFI in the developing dorsal telencephalon is also seen caudally (yellow arrowhead in B').

At E12.5, *COUP-TFI*^{wt} brains have *COUP-TFI* expressed in the preplate and ventricular zone of the pallium, with strongest expression being in the ventricular zone ventrally and caudally. At rostral levels, COUP-TFI expression extends from the pallial-subpallial boundary dorsally in a gradient fashion, but is not seen very dorsally, while at caudal levels COUP-TFI expression can be detected extending into the cortical hem (Fig. 8, left column). Expression at the pallial-subpallial boundary is continuous with expression in the subpallial proliferative zones of the lateral and medial ganglionic eminences, and at caudal levels the caudal ganglionic eminence and most of the subpallium appear COUP-TFI positive. The conditional mouse displays a complete loss of COUP-TFI from the cortex (Fig. 8, yellow arrows), while expression in subpallial regions is not changed.

At later stages, such as E13.5 and E15.5 wild-type COUP-TFI expression is extended to the newly formed cortical plate, subventricular and intermediate zones. At 13.5 the expression is weaker in the cortical plate than in the ventricular zone, and at E15.5 the expression is particularly strong in the ventricular zone, with intermediate levels seen in the marginal zone and cortical plate, and lowest expression in the intermediate zone. Similar to E12.5, the most dorsal and medial extension of expression within the cortex is detected caudally. Outside of the dorsal telencephalon, COUP-TFI protein is detected in the lateral and medial ganglionic eminences, as well as in the preoptic area and caudally throughout the basal telencephalon, including the regions around the internal capsule. A migrating stream of COUP-TFI-positive cells appears to mark the pallial-subpallial boundary and might correspond to the radial migratory stream , as previously described (Medina et al., 2004). In the diencephalon, expression is seen in the dorsal thalamus starting at E13.5. At these stages, the conditional tissue displays a complete loss of COUP-TFI in cells generated within the

cortex, such that the cortical plate and ventricular zone do not express COUP-TFI, while two migratory streams from the subpallium directed towards the intermediate and marginal zones result in few COUP-TFI positive cells detectable in the cortex (Fig. 8, blue arrow). At E12.5, the cells have not yet exited the basal telencephalon, and can be detected in the ganglionic eminences, such that the cortex at this stage is genuinely COUP-TFI negative. These migratory streams have been described as being Calbindin- and GABA-positive (Tripodi et al., 2004) constituting a population of GABAergic interneurons which is outside the *Emx1*-lineage and hence not subject to the action of CRE-recombinase on the floxed exon3 of *COUP-TFI* (Gorski et al., 2002). The medial and ganglionic eminences continue to express COUP-TFI, as do the caudal ganglionic eminence, the presumptive amygdala and the thalamus. Staining at E15.5 displays the same cortex-specific loss of COUP-TFI. A confirmation of the loss of expression of COUP-TFI in the *COUP-TFI*^{null} mutant was also carried out by immunofluorescence (Fig. 9A). In the *COUP-TFI*^{null} mutant the expression of COUP-TFI seen in the wild type cortex, subpallium and thalamus is lost (Fig. 9, A' compared to A), as would be expected from a complete knock-out COUP-TFI embryo. This confirms that the null mutant can be used as a model in which to study development in the absence of COUP-TFI from all tissues.

The earliest telencephalic expression of COUP-TFI during development has been detected at E9.5 in caudalmost regions (M. Studer, personal communication). Here we have confirmed the activity of CRE-recombinase in the conditional at E10.5 (Fig. 7). This suggests that in the conditional mutant COUP-TFI is acting during cortical development for one day before Cre expression is turned on. Hence these data indicate that COUP-TFI is removed from cells generated within the dorsal

telencephalon, and that conditional mutant mouse may be used as a model for the effects of COUP-TFI in the cortex in particular.

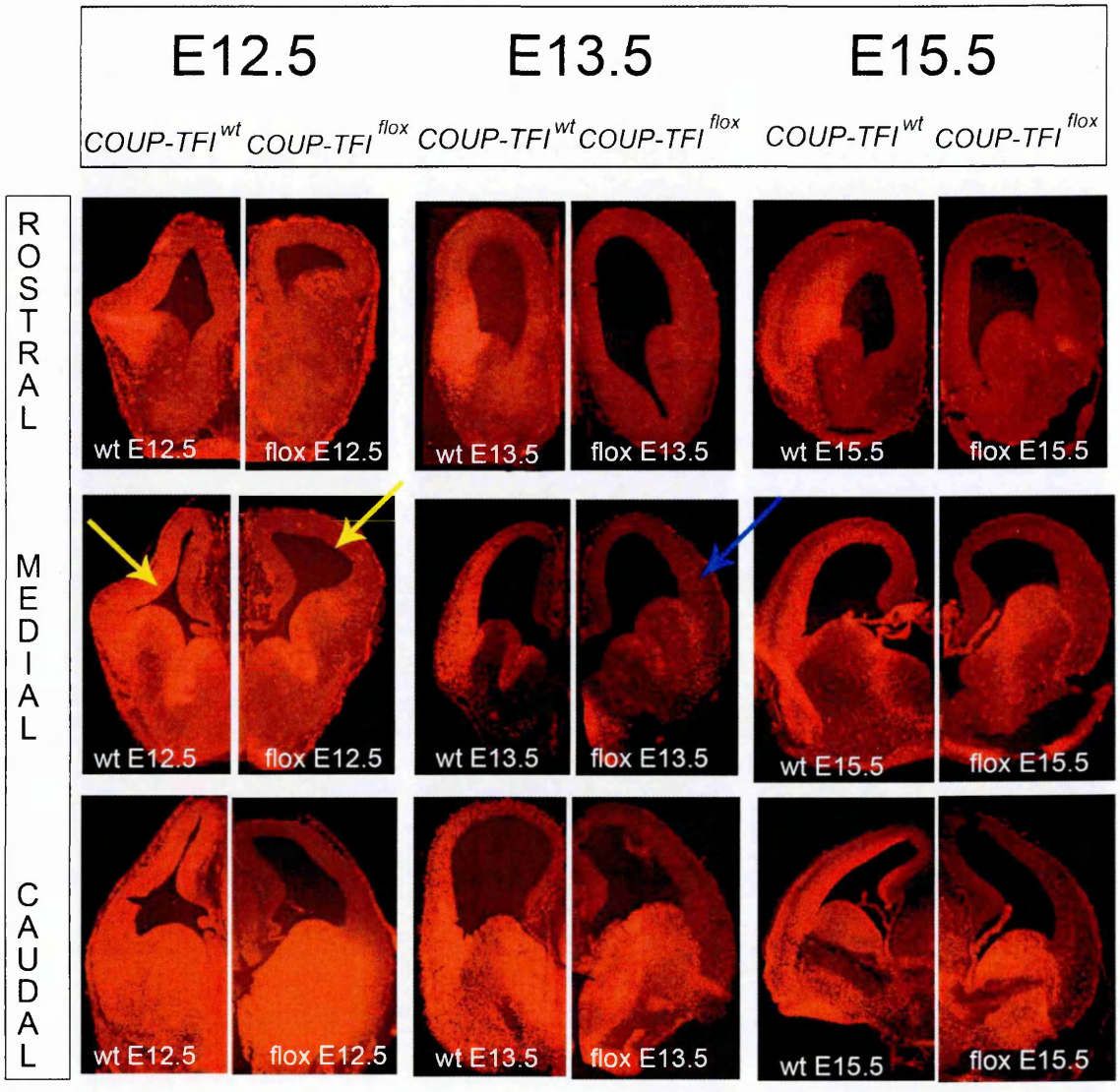


Fig. 8. Sections of *COUP-TFI*^{wt} (hemisections on left of every image) and *COUP-TFI*^{flox} (right) brains at E12.5, E13.5 and E15.5 at three rostrocaudal levels. *COUP-TFI* expression is lost in the dorsal telencephalon of *COUP-TFI*^{flox} mutants (yellow arrows), while expression is maintained in neurons outside the *Emx1* lineage migrating from the basal telencephalon to the cortex (blue arrow).

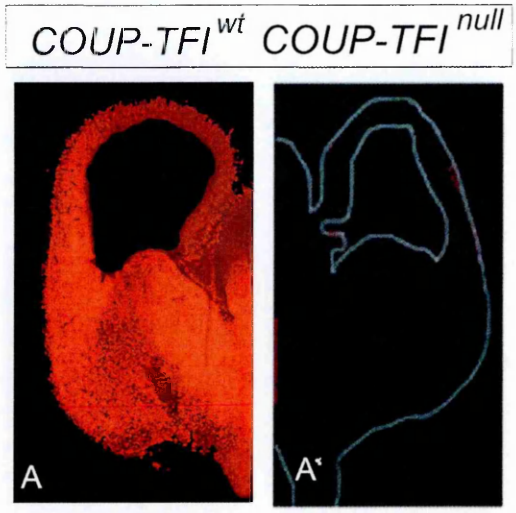


Fig. 9: The COUP-TFI null mutant displays a loss of expression of COUP throughout the brain (A' compared to A).

2. Proliferation and Early Neurogenesis

As COUP-TFI has previously been implicated in regulating neuronal differentiation *in vitro* (Adam et al., 2000; Studer et al., 2005), we aimed to investigate whether the balance between proliferation and differentiation was disrupted *in vivo.* To do this we used markers of neural progenitors in the ventricular and subventricular zones, such as Ngn2, Gli3, Tis21, Tbr2, Svet1 and Cux2 at E12.5 and/or E13.5, and markers for proliferating neurons, such as short pulses of BrDU and Phosphohistone H3 for mitotic cells. Using these markers we characterized a phenotype that suggests increased proliferation and decreased differentiation at early stages of cortical development in the absence of COUP-TFI.

To study the rate of proliferating cells in the neuroepithelium, we first used Phosphohistone H3 at E12.5, which labels a layer of mitotic cells in the apical ventricular zone and sparsely distributed mitotic cells in the basal ventricular zone (Fig. 10, A-D). In *COUP-TFI*^{flox} embryos of the same stage, this pattern of distribution of mitotic cells is seen to be altered, with an increased number of progenitors placed in both the basal and apical ventricular zone (Fig. 10, red arrows in A'-D'). At E15.5, we continue to see an altered distribution, however, it primarily affects the basal ventricular zone at this stage (Fig. 11, red arrows in A'-C').

Next, we injected BrdU at E13.5 and stained for BrdU immunoreactivity 3 hours after injection to assess the number of cells in S-phase in wild-type and COUP-TFI^{flox} animals. In both wild-type and *COUP-TFI*^{flox} sections, the population of cells in S-phase is mainly restricted to the ventricular and subventricular zones. However, in *COUP-TFI*^{flox} mutants, the level of staining is slightly higher, in particular at caudal

levels, suggesting that more cells are proliferating in the absence of COUP-TFI (Fig. 12, yellow arrow in B').

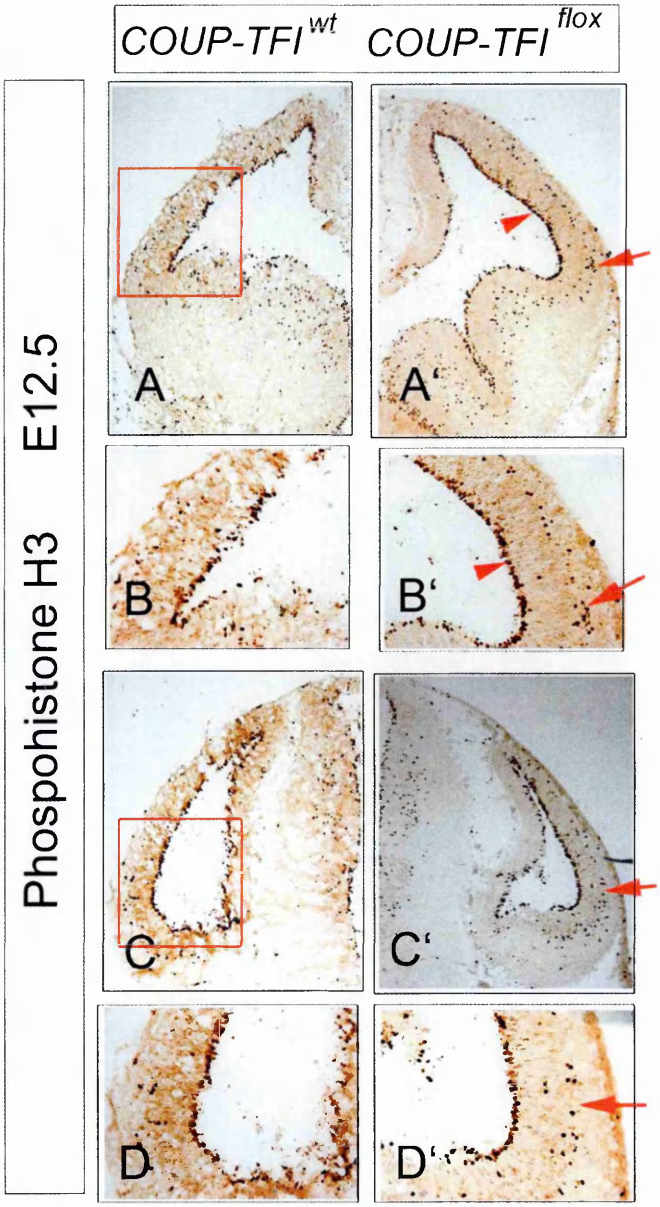


Fig. 10. Progenitors undergoing mitosis in the basal ventricular zone are marked by Phosphohistone H3. In wild-type sections this marker primarily marks the apical ventricular zone and sparse cells in the basal ventricular zone at E12.5 (A-D); in the *COUP-TFI*^{flox} mutant, the number of cells that are Phosphohistone H3-positive appears increased (red arrows in B' and D').

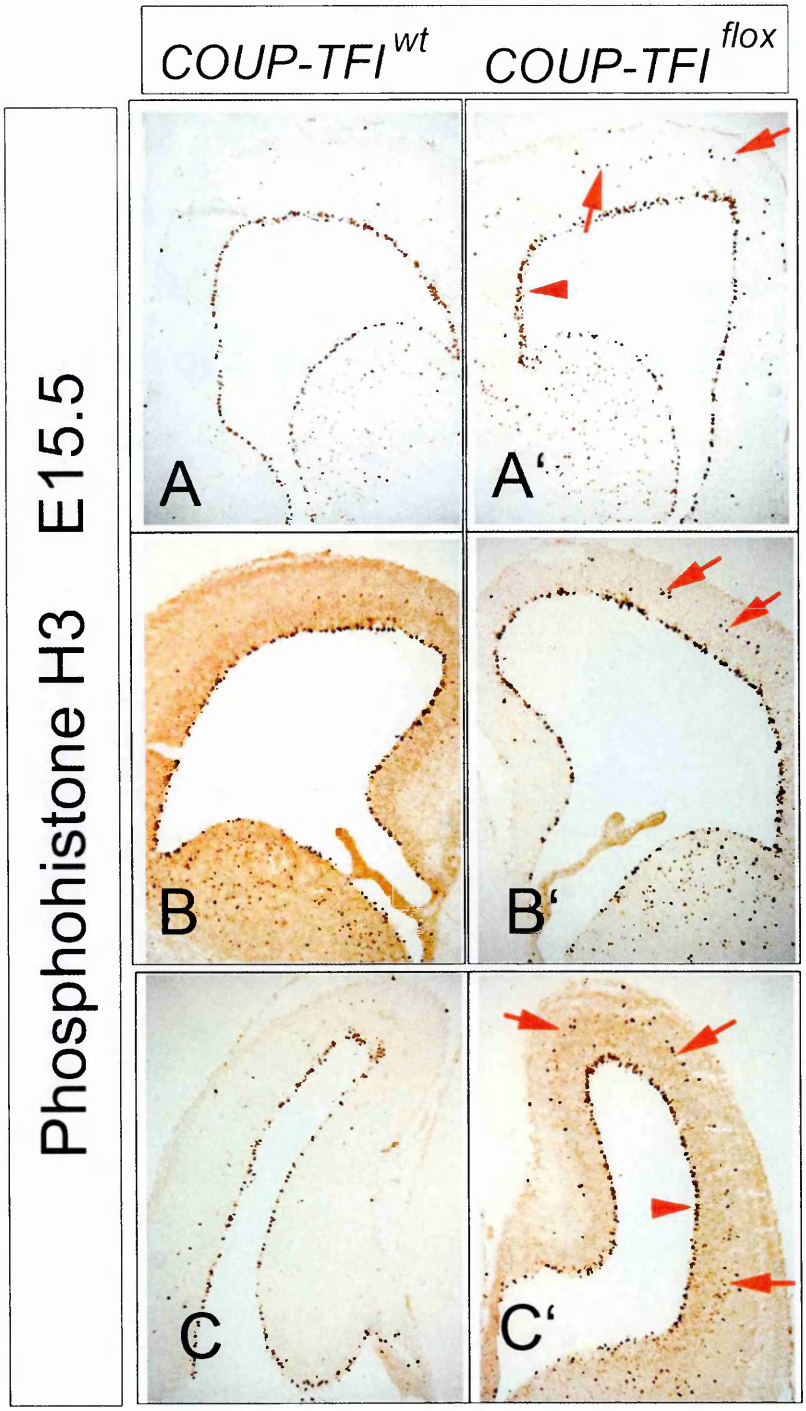


Fig. 11. Phosphoshistone H3 staining at E15.5 reveals an increased number of mitotic cells in the basal ventricular zone in *COUP-TFI*^{flox} mutants (red arrows in A'-C').

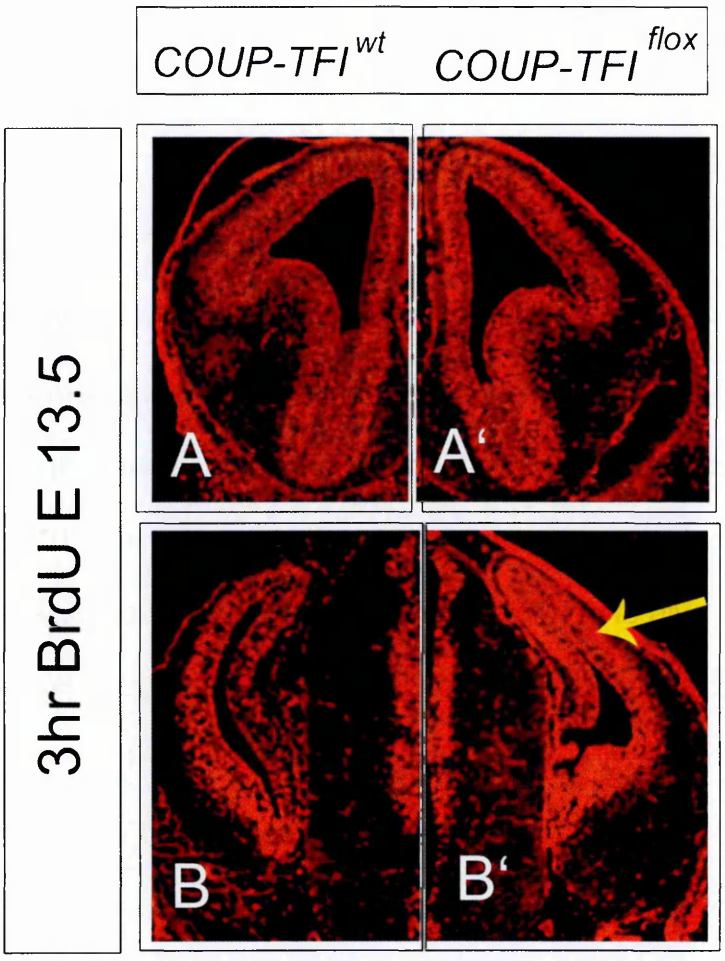


Fig. 12. BrdU injections at E13.5 3 hours before staining: BrdU is integrated into cells in S-phase in the ventricular zone of wild-type animals (A, B). *COUP-TFI*^{flox} mutants show an increase in staining for BrdU suggesting increased number of cycling cells in the ventricular zone (yellow arrow in B'). This is more evident at caudal levels.

To understand whether the number of intermediate progenitors was also increased in the absence of COUP-TFI, we used Tbr2, a member of the T-box family, which marks preplate neurons from E10.5 – E12.5 (see also Results section 3. Preplate Development), but also intermediate progenitors in the ventricular zone from E11.5 onwards (Englund et al., 2005). The expression of Tbr2 in wild-type sections at E12.5 can be clearly seen in early preplate neurons in the dorsal pallium, while its

expression in intermediate progenitors can be better appreciated in the ventral pallium in cells located in the ventricular zone (Fig. 13, A). In COUP-TFI^{flox} and null mutants, the number of cells in the intermediate progenitor class appears increased (Fig. 13, A', B', C'). Furthermore, *COUP-TFI^{flox}* and null mutants show an ectopic patch of putative intermediate progenitors in the presumptive pyriform cortex (Fig. 13, arrows in B, C). These data suggest that the loss of COUP-TFI causes an increase of intermediate or basal progenitors in the neuroepithelium during early cortical development.

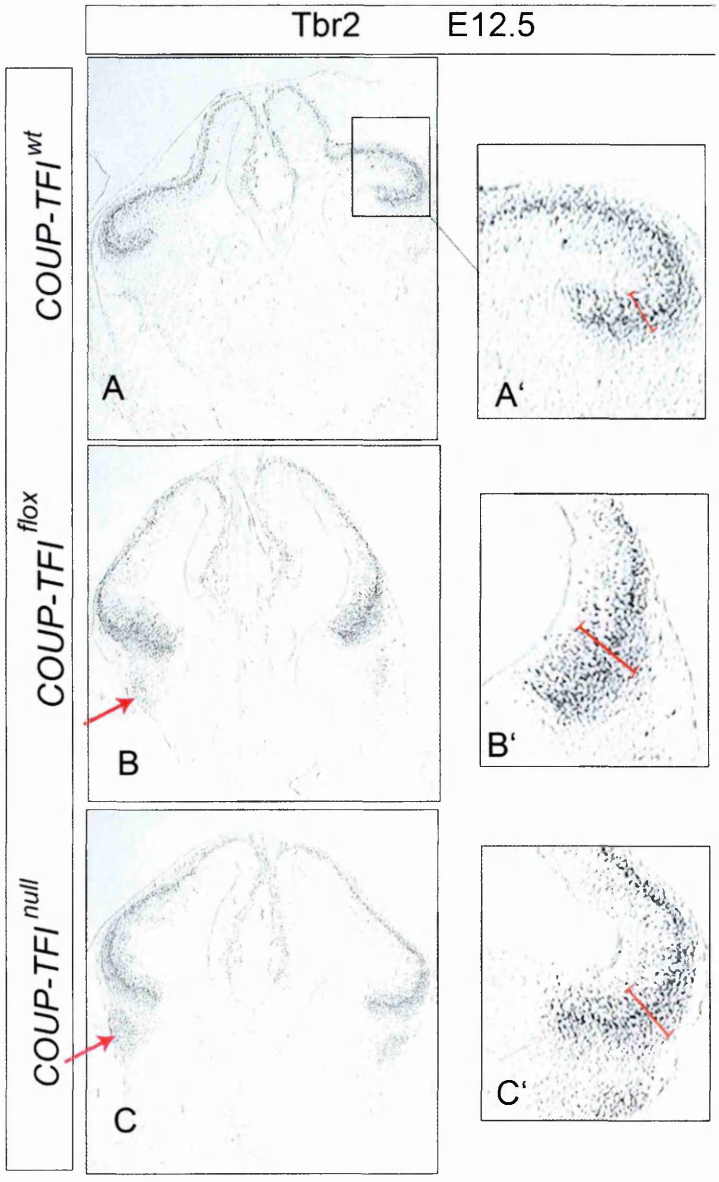


Fig. 13: At E12.5, Tbr2 labels intermediate progenitors exiting from the neuroepithelium in the ventral pallium. In the absence of COUP-TFI it appears that the population of intermediate progenitors is increased (red bars in A'-C', and and an ectopic patch of putative intermediate progenitors is visible (red arrows, A-C).

The markers *Ngn2*, *Gli3* and *Tis21* are all genes expressed in the ventricular zone that promote neurogenesis. *Ngn2* is a proneural gene of the bHLH class that has been implicated in the determination of neuronal fate in the cortex (Mattar et al., 2004). *Gli3* is a homolog of the *Drosophila* gene *cubitus interruptus* and has been shown to regulate Wnt signalling in the dorsal telencephalon (Grove et al., 1998). Its expression in the pallium is required for the apical/basal cell polarity of cortical progenitors and for the differentiation of early-born cortical neurons (Theil, 2005). *Tis21* is expressed in the ventricular and subventricular zones and is a marker that is specific to basal progenitors that will divide to give rise to neurons (Haubensak et al., 2004). *Tis21* mRNA expression in this subpopulation of progenitors is restricted to G1 phase of the cell cycle, while the protein is expressed throughout the cell cycle and is inherited by neurons (Iacopetti et al., 1999). The expression levels of these neurogenic markers in wild-type sections are shown in the figure below (Fig. 14, A-F). Comparison of wild-type with *COUP-TFI*^{flox} tissue reveals that the expression is up-regulated in the absence of COUP-TFI, suggesting an increase in the population of neuron-generating progenitors (Fig. 14, red arrows in A'-F').

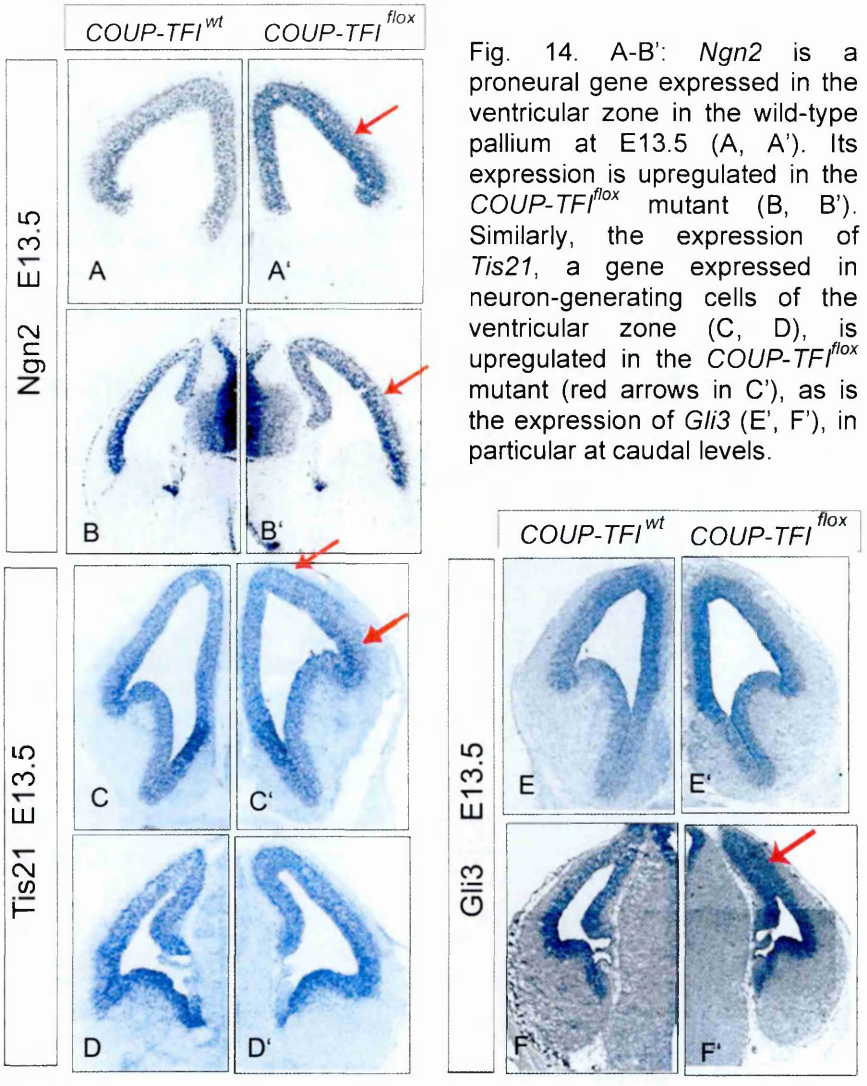


Fig. 14. A-B': *Ngn2* is a proneural gene expressed in the ventricular zone in the wild-type pallium at E13.5 (A, A'). Its expression is upregulated in the *COUP-TFI*^{flox} mutant (B, B'). Similarly, the expression of *Tis21*, a gene expressed in neuron-generating cells of the ventricular zone (C, D), is upregulated in the *COUP-TFI*^{flox} mutant (red arrows in C'), as is the expression of *Gli3* (E', F'), in particular at caudal levels.

We next inspected the expression of markers of the subventricular zone at E13.5. *Cux2* is a homolog of *Drosophila* gene *cut* that is expressed exclusively in the subventricular zone of proliferating cells. It has been suggested that progenitors expressing *Cux2* are a pool of early progenitors that give rise to upper layer cortical neurons (Zimmer et al., 2004). *Svet1* similarly is a marker of the subventricular zone that labels a population of progenitors which give rise to upper layer neurons (Tarabykin et al., 2001). Comparing the expression of these markers in wild-type sections (Fig. 15, A-D), with *COUP-TFI*^{flox} mutant tissue reveals increased expression of both markers at the ventricular zone (Fig. 15, red arrows A'-D').

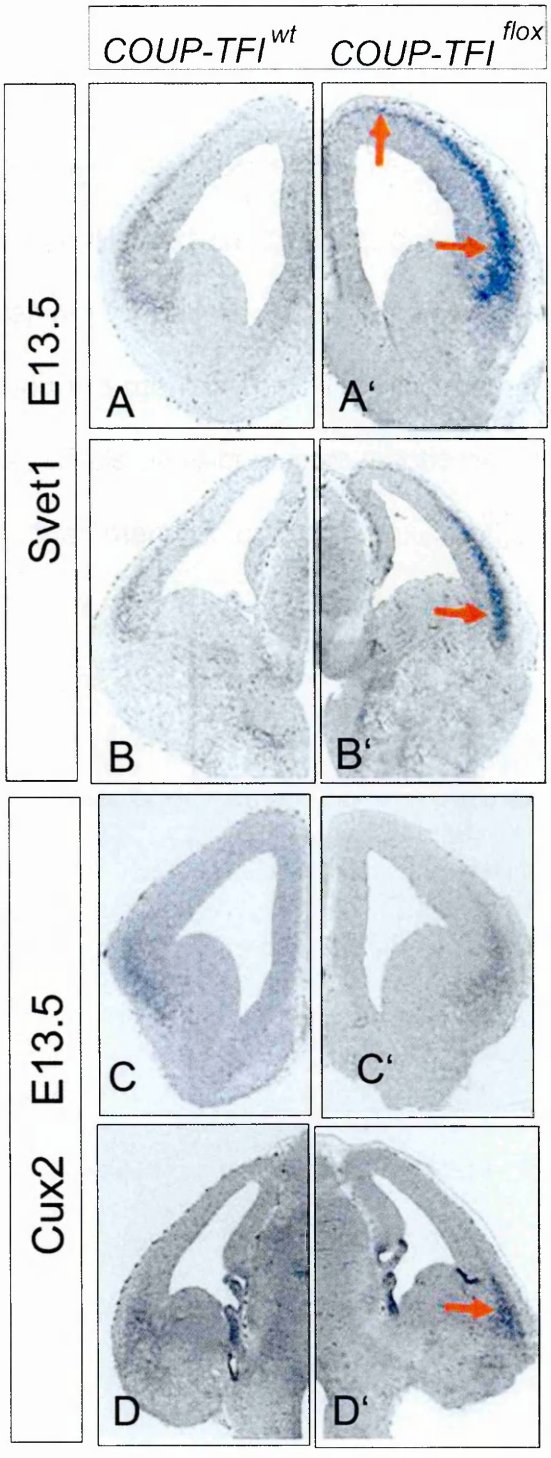


Fig. 15. Expression of markers of the subventricular zone. *Svet1* and *Cux2*, markers of the subventricular zone display increased expression in the subventricular zone of *COUP-TFI*^{flox} mutants (red arrows in A',-D').

These stainings indicate that proliferation in the ventricular and subventricular zones is increased in the *COUP-TFI*^{flox} mutant. Depending on a balance struck between proneural and neurogenic genes in the proliferative layers, these changes could lead either to increased or decreased production of neurons. To investigate the effects of these changes during corticogenesis, we assessed changes in markers for preplate and subplate neurons, followed by neurons of the cortical plate.

3. Preplate Development

A previous report has shown that in the absence of COUP-TFI, the subplate is abnormally formed (Zhou et al., 1999). To understand whether this defect could arise upon subplate formation, i.e. when cortical plate neurons split the preplate into marginal zone and subplate, or whether the defect is due to intrinsic problems in early preplate formation, we assessed the development of the preplate at E12.5 and E13.5 using the marker Tbr1. Tbr1 is a member of the T-box family of transcription factors that labels early-born post.mitotic neurons (Bulfone et al., 1995). We also used Tbr2, another member of the T-box family, which is a marker of preplate neurons from E10.5 to E12.5, as well as a marker of intermediate progenitors from E11.5 onwards (Englund et al., 2005).

Wild-type cortex at E12.5 is characterized by a single layer, the preplate, which contains Tbr1-positive neurons. This layer is seen to be approximately 5 cells thick and continuous in wild-type (Fig.16, A, A'), while both *COUP-TFI*^{flox} mice and *COUP-TFI*^{null} mice show an altered pattern of Tbr1 expression. In *COUP-TFI*^{flox} and *COUP-TFI*^{null} mice, the preplate is thinner, and is not a continuous layer of cells but is characterized by gaps, such that overall fewer cells appear to make up the *COUP-TFI*^{flox} and *COUP-TFI*^{null} preplates compared to wild-type (Fig. 16, B, B', C, C', red arrows indicate gaps). Preliminary counts on the ventral and dorsal pallium confirm a decrease of Tbr1-positive neurons mainly in the dorsal pallium, which is present in both models, the null and the *COUP-TFI*^{flox} mutants; these changes are statistically significant (Fig.17). The finding that the preplate is affected was confirmed also by *in situ* hybridization at E13.5 (Fig. 18). The preplate and nascent cortical plate are Tbr-1 positive in wild-type sections. In *COUP-TFI*^{flox} mutants,

staining in the preplate, identified as a layer at the superficial surface of the cortex is decreased, (Fig. 18, red arrow in C').

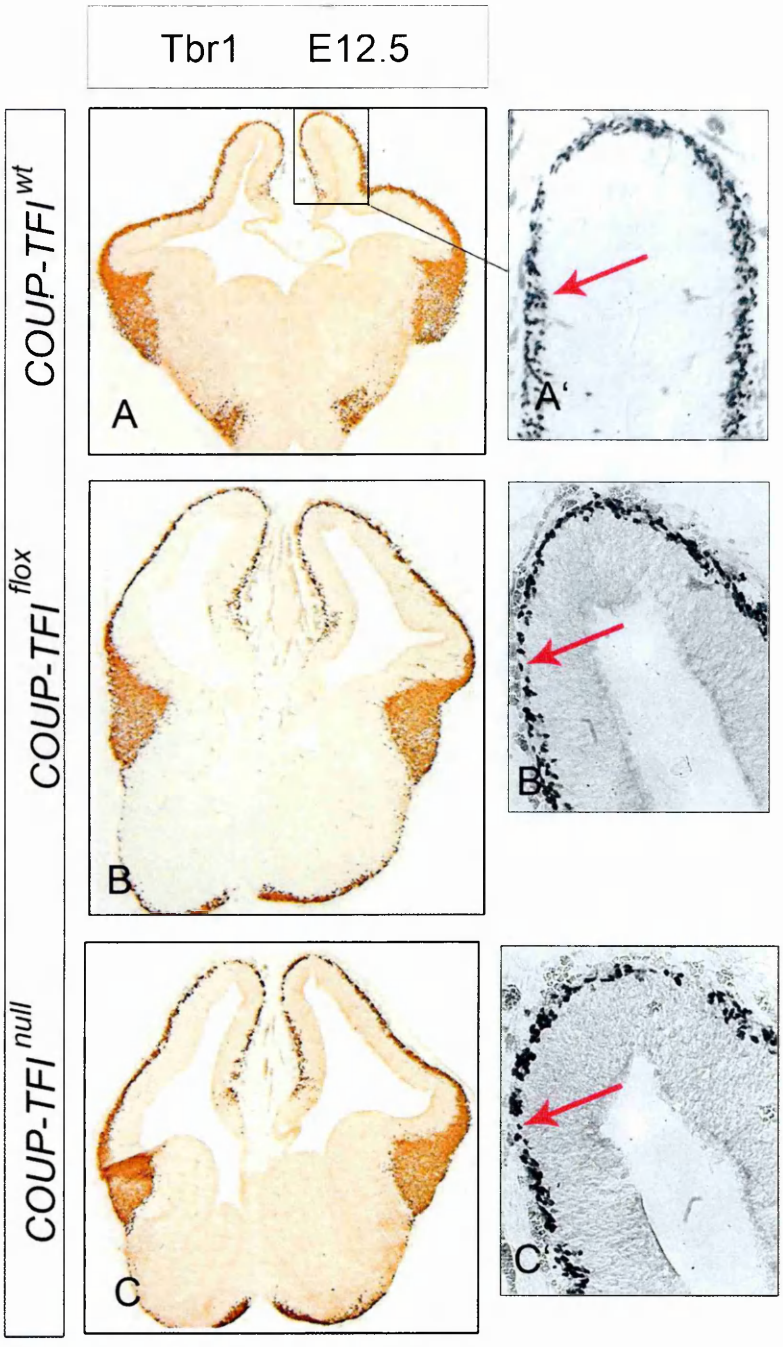


Fig. 16. Tbr1 marks early born neurons in the preplate layer at E12.5 (A and A'). This layer is seen to be thinner and contains gaps in both *COUP-TFI*^{null} and *COUP-TFI*^{flox} mutants (red arrows in B' and C').

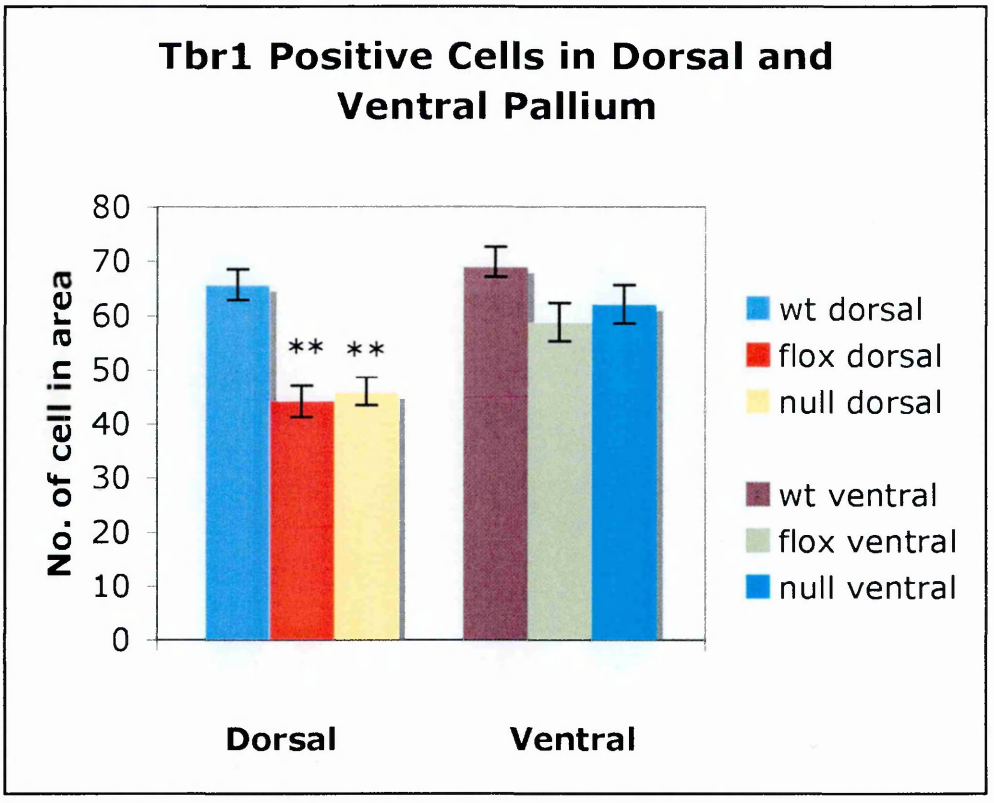


Fig. 17. Counts of Tbr-1 positive neurons in the dorsal and ventral pallium counted in a fixed area at E12.5 suggest that the preplate contains a significantly decreased number of neurons in *both COUP-TFI*^{flox} and *COUP-TFI*^{null} mutants (dorsal: wt mean: 63, SEM: 2.9; flox mean: 44, SEM: 1.8; null mean: 47, SEM: 2.4. ventral: wt mean: 67, SEM: 3.7; flox mean: 59, SEM: 3.5; null mean: 60, SEM: 3.5).

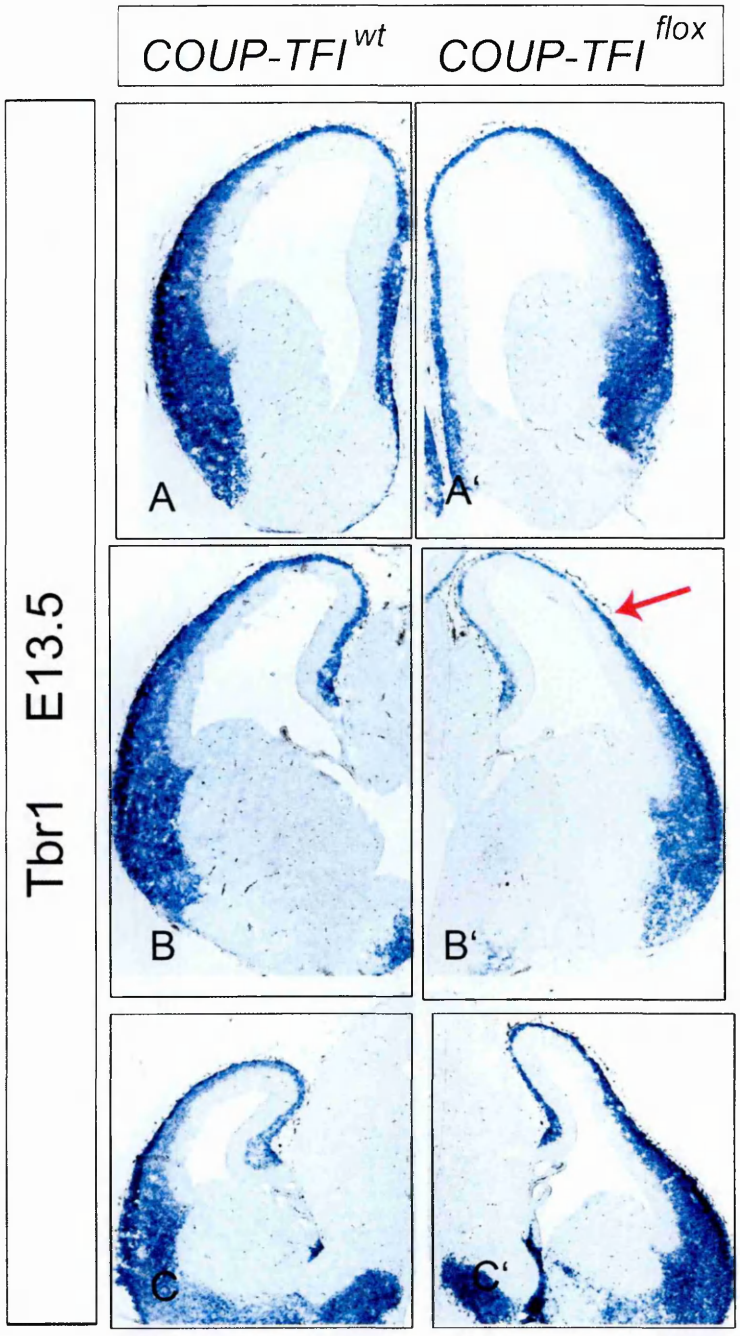


Fig. 18. *In situ* hybridization for Tbr1 labels postmitotic neurons in the preplate and nascent cortical plate of wild-type tissue at E13.5 (A-C). The layer of postmitotic neurons is diminished in *COUP-TFI*^{flox} sections (A'-C', red arrow in B').

A second marker, Tbr2, was used to confirm this finding. Tbr2 at E12.5 labels differentiated preplate neurons and intermediate progenitors in the ventricular zone (Fig. 19). In wild-type embryos, Tbr2 labels early-born preplate cells, similarly to Tbr1 (Fig. 19, A, A'). However, in *COUP-TFI*^{flox} and *COUP-TFI*^{null} cortices, the preplate shows the same thinned, discontinuous structure seen with Tbr1 (Fig. 19, green arrows in B', C').

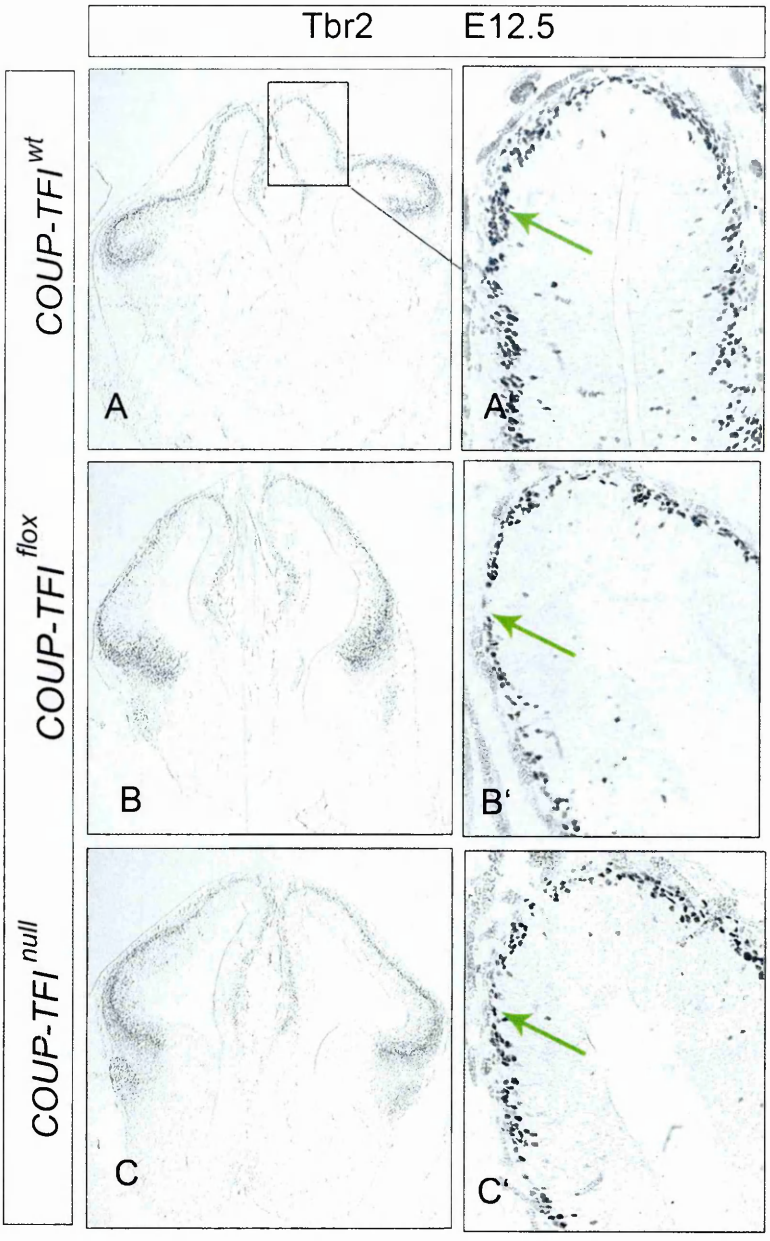


Fig. 19. At E12.5, Tbr2 is a marker of preplate neurons in the dorsal pallium (green arrow in A'). Like Tbr1, Tbr2 shows a thinner and discontinuous preplate layer in the absence of COUP-TFI (green arrows in B' and C').

These data suggest a decrease in the number of early-born preplate neurons that are generated in the absence of COUP-TFI. The defects seen in the *COUP-TFI*^{null} mutant and *COUP-TFI*^{flox} mutant are comparable, suggesting that the *COUP-TFI*^{null} mutant phenotype in the preplate results from an intrinsic requirement for COUP-TFI in the cortex. Given the increases in markers of the ventricular zone described above, it appears that the loss of COUP-TFI results in an increase in proliferation and a decrease in early differentiation.

4. Cortical Development: Subplate Formation

In the previous chapter we found a defect in preplate development that expressed itself as a decreased number of differentiated neurons. To understand whether the derivative of the preplate, the subplate, is normally differentiated in the absence of COUP-TFI, we analyzed subplate markers in *COUP-TFI*^{flox} mutant embryos, where COUP-TFI is inactivated solely in the dorsal telencephalon. It was previously postulated that in the complete absence of COUP-TFI, thalamocortical axon pathfinding defect was a consequence of misdifferentiation of subplate neurons, which serve as an intermediated target for TCAs before innervating the cortical plate (Zhou et al., 1999, 2001). The subplate was reported to form normally, but not to express the full complement of markers present in wild-type embryos, and was subject to early degeneration. The thalamocortical pathfinding defect in turn was identified as the cause of loss of layer IV neurons. To first investigate whether a causal link could exist between the cortical and thalamocortical defects described, we compared the development of the subplate in *COUP-TFI*^{flox} and *COUP-TFI*^{null} mutants. The subplate can first be identified at E13.5, when the preplate is split into the marginal zone and subplate by incoming cortical plate neurons, and persists until birth, after which the majority of subplate neurons die, but some are maintained as layer VIb (Ferrer et al., 1990). The markers Calretinin, CSPG, *Sema6a* as well as Nissl staining were used to assess the state of the subplate at different developmental stages.

The calcium-binding protein Calretinin at E13.5 primarily marks the marginal zone (Fonseca et al., 1995), but the nascent subplate can be identified by the presence of individual cells lying basally with respect to the cortical plate (Fig. 20, red arrows in

C). These cells could not be clearly identified in either the *COUP-TFI*^{flox} or *COUP-TFI*^{null} mutant (Fig. 20, B, D).

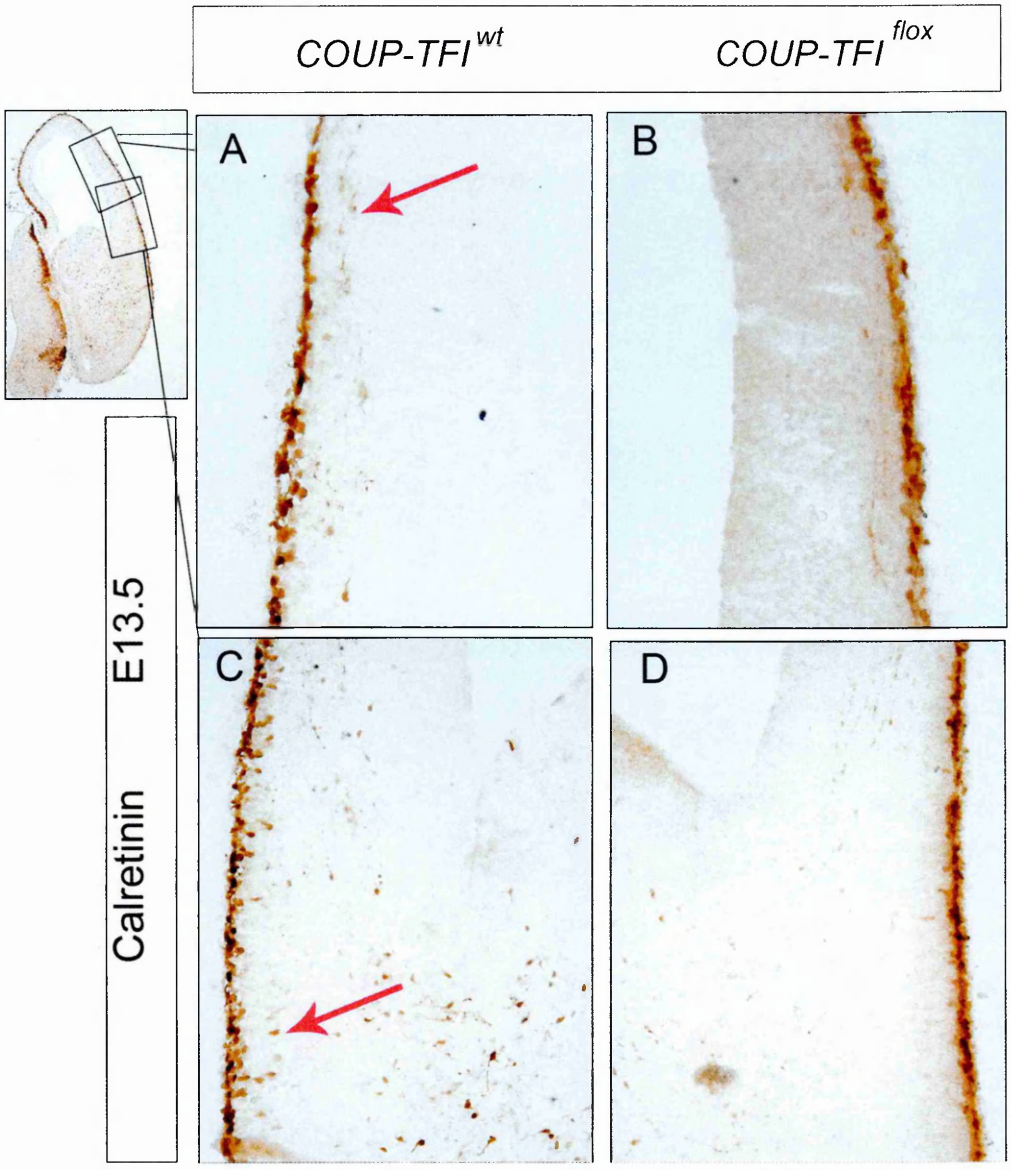


Fig. 20. In wild-type brain at E13.5 Calretinin marks a subpopulation of cells within the subplate (red arrows in A, C). While Calretinin positive cells can be detected within the marginal zone, no Calretinin staining is seen within the subplate of *COUP-TFI*^{flox} mutants, suggesting a defect in subplate development.

To confirm the Calretinin result, we examined the distribution of Chondroitin sulfate proteoglycans (CSPG), which are closely associated with the preplate and preplate-

derived marginal zone and subplate (Bicknese et al., 1994). At E13.5 CSPG-positive cells are clearly identified in the marginal zone and in the nascent subplate cells, which are more abundant in ventral regions, due to the neurogenic gradient (Fig. 21 A, A'). In *COUP-TFI*^{flox} embryos, CSPG-positive cells are present in the subplate, but they look more dispersed and do not reach the more dorsal regions (Fig. 21, B, B', arrowhead).

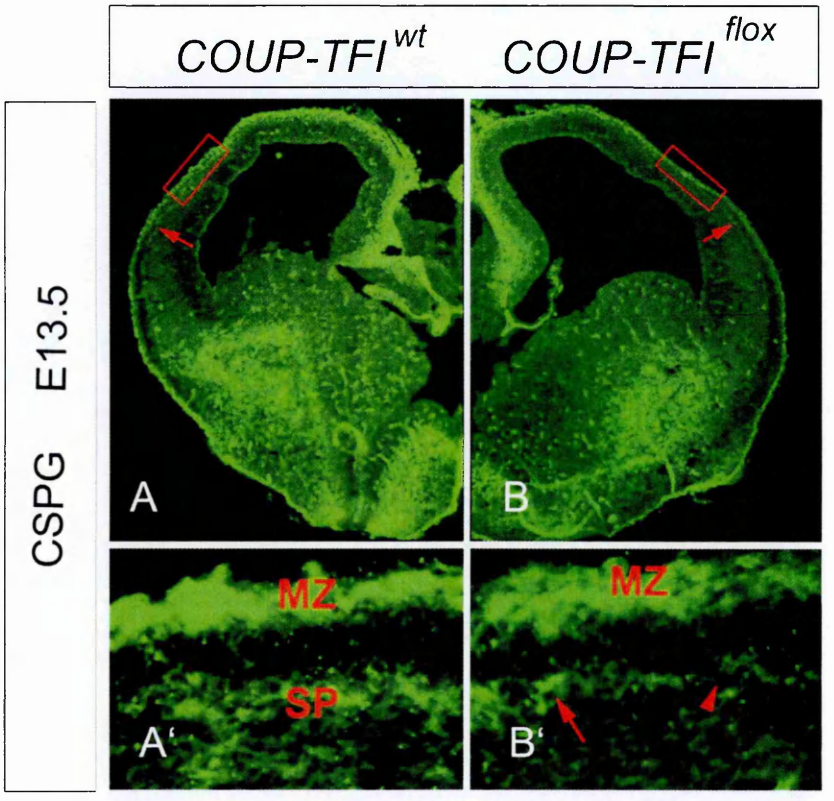


Fig. 21. CSPG staining at E13.5 in wild-type and *COUP-TFI*^{flox} sections show expression in the subplate of wild-type tissue (A, A'), but decreased expression in the *COUP-TFI*^{flox} mutant (B, B').

At E15.5, the subplate is more clearly distinguished than at E13.5 as the cortical plate has expanded and has split the preplate at all dorso-ventral levels in the cortex. At this stage Tbr1 labels a discrete subplate cell layer in the wild-type (Fig. 22, red arrows in A', C'), but not in *COUP-TFI*^{flox} mutants, which show a diminished and

disorganized subplate layer (Fig. 22, red arrows in B', D').

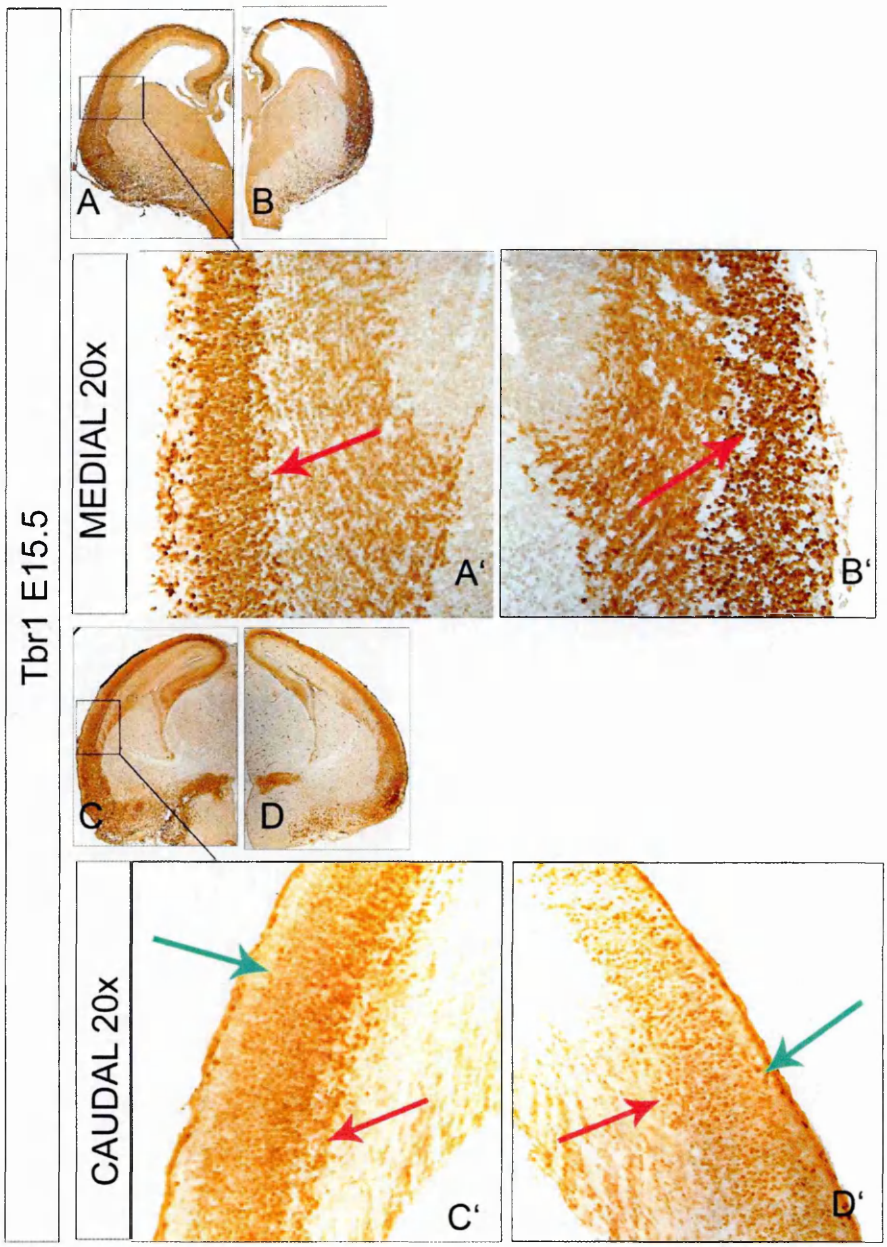


Fig. 22. Tbr1 staining at E15.5 labels neurons of the subplate (red arrows in A', C') and cortical plate. In the *COUP-TFI*^{flox} mutant, the subplate is not clearly distinguished from the cortical plate (red arrow in D') and its absence even produced irregular gaps at some locations (red arrow in B'). Tbr1 staining is stronger in neurons with an earlier birthdate, in the subplate and future deep layers compared to more superficial neurons (green arrow in C'). This gradient of staining is not apparent in the *COUP-TFI*^{flox} mutant, where more strongly labeled cells are also apparent at the radial surface of the cortical plate (green arrow in D').

In wild-type cortex at E15.5, the subplate can also be distinguished as a thin layer of cells expressing *Sema6A*, a guidance molecule of the semaphorin family (Zhou et al., 1997) (Fig. 23, A,B). In the *COUP-TFI*^{null} mutant however, this layer is much fainter, suggesting that the subplate either has lost expression of *Sema6A* in particular, or is

itself decreased (Fig. 23, red arrows in A', B').

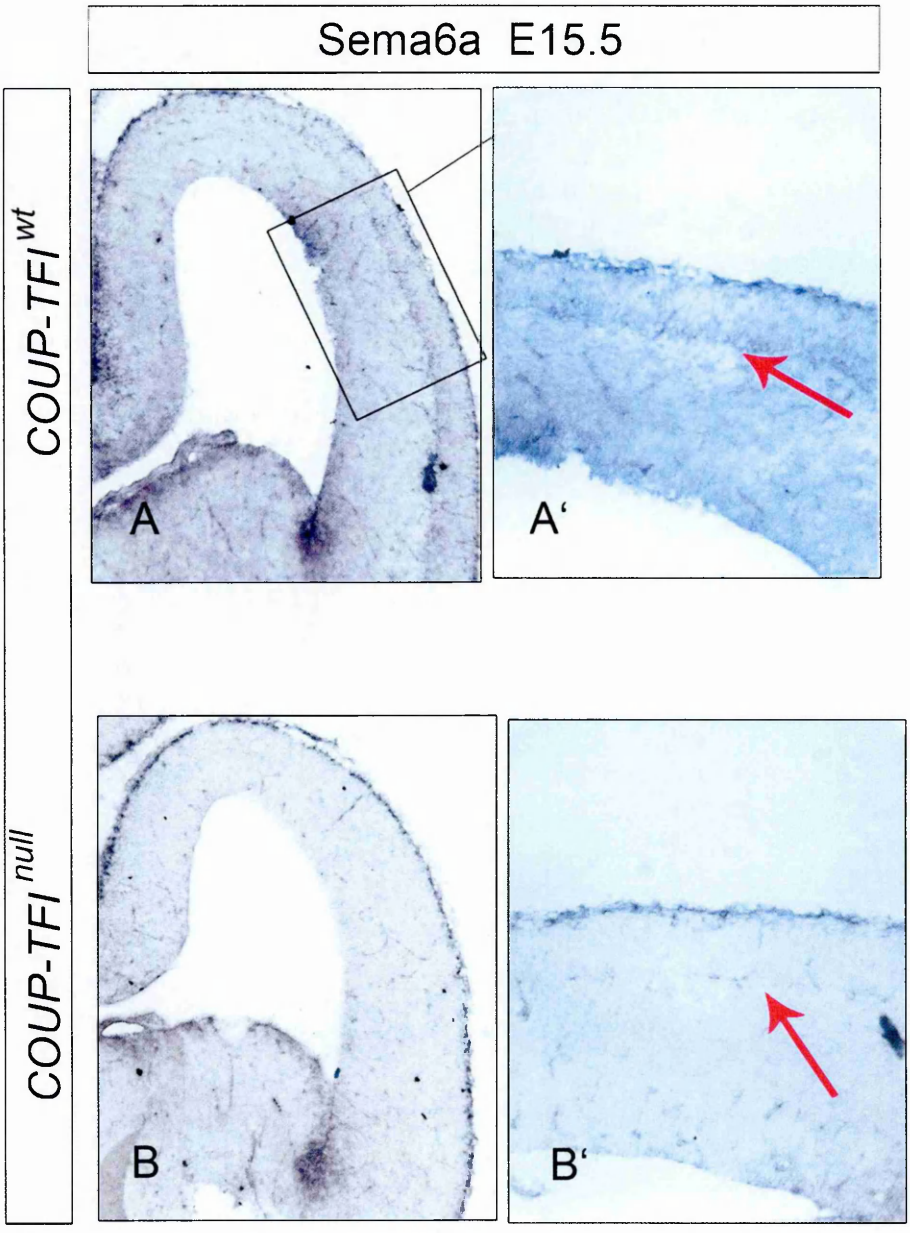


Figure 23: *In situ* hybridization for *Sema6a* in *COUP-TFI*^{wt} (A) and *COUP-TFI*^{null} mutant (B) at E15.5 revealed a loss of *Sema6a* expression in the subplate in the *COUP-TFI*^{null} mutant (red arrows A', B').

Finally, we investigated the fate of subplate cells at perinatal stages. Nissl staining show that at E18.5 and P0, the *COUP-TFI*^{flox} mutants lack a distinguishable subplate layer, differently from wild-type embryos in which the subplate layer can be easily identified as a continuum of cells (see Fig. 24, Results section 5. Development of the Cortical Layers). The fact that a number of markers for different genes and proteins expressed in the subplate are affected from E13.5 onwards, suggests that the subplate itself is abnormal from the beginning rather than simply not expressing individual markers. Finally, Nissl staining at E18.5 and P0 shows an intact subplate in wild-type cortex but in both *COUP-TFI*^{null} at E18.5 and *COUP-TFI*^{flox} at P0 the subplate is less clearly formed and appears disorganized and/or strongly diminished. This suggests that the defect seen in the *COUP-TFI*^{null} mutant subplate is due to a cortical requirement for COUP-TFI during early neurogenesis. However, the absence of subplate cells at later stages might be due to a role for COUP-TFI in maintaining subplate differentiation.

5. Development of the Cortical Layers

Given the early defects in neurogenesis we detected, we aimed to investigate whether the cortical plate was correctly formed. To address this question, we investigated the layers of the cortical plate between E18.5 and P0. First, general cortical architecture was assessed using Nissl staining. Then, the markers Tbr1, *Foxp2* and *Fez* were used to assess lower layers (layer V and VI), while *ROR-ß,* and *SCIP* were used to assess upper layers (Layers IV and II/III, respectively). Finally, BrdU birthdating experiments were performed to establish whether radial cortical migration was affected.

Nissl staining was carried out in wild-type and *COUP-TFI*^{flox} mutants at P0 and in *COUP-TFI*^{null} mutants at E18.5. In wild-type sections at E18.5 and P0, the cortex can be divided into marginal zone, cortical plate, subplate, intermediate zone and subventricular and ventricular zones (Fig. 24, A, A', C, C'). In *COUP-TFI*^{null} and *COUP-TFI*^{flox} mutants, all of these structures, with the exception of the subplate, can be recognized as being substantially present, although they show a disorganized pattern (Fig. 24, red arrows in B, B', D, D'). Hence, it appears that there are defects in cortical development that reach beyond the altered subplate described. However, Nissl staining is not sufficient to allow us to distinguish well between the layers of the cortical plate, such as to localize which parts of the cortex are most affected, and the extent of the defects. To further investigate the defects resulting from loss of COUP-TFI, we used the markers described above above at P8, when upper and lower layers can be better distinguished.

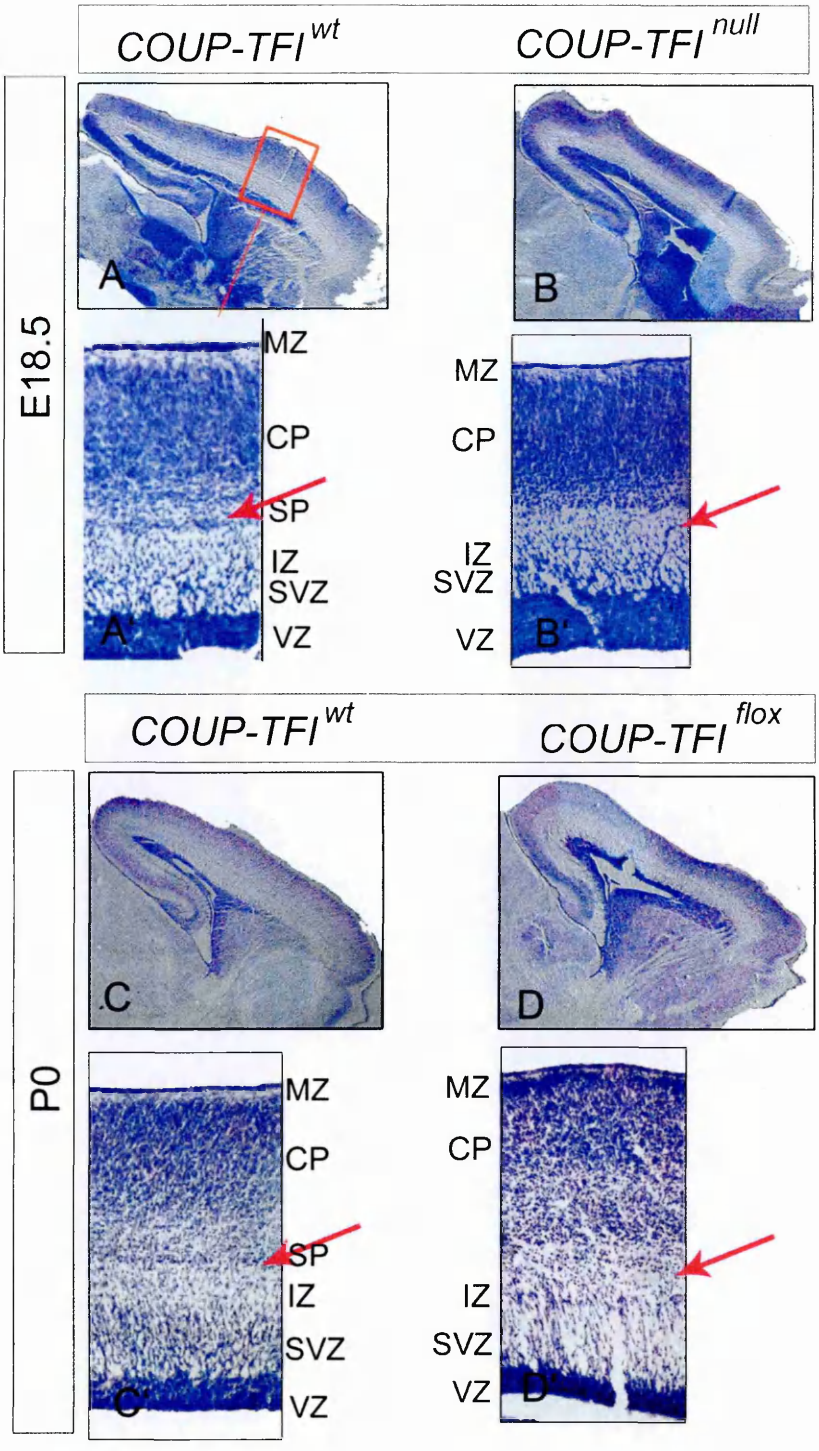


Fig. 24. Nissl staining in *COUP-TFI*^{wt} and *COUP-TFI*^{flox} at E18.5 and P0. *COUP-TFI*^{flox} and *COUP-TFI*^{null} mice show a decreased subplate (red arrows), and changes in general cortical architecture. VZ: ventricular zone, SVZ: subventricular zone, IZ: intermediate zone, CP: cortical plate, MZ: marginal zone.

The use of Tbr1 as a marker for post-mitotic neurons has been described above. During layer formation, Tbr1 has been considered primarily a layer VI marker, although lower expression is also observed in layers II/III (Bulfone et al., 1995 and Fig. 25). At P8 in *COUP-TFI*^{flox} mutants, it is apparent that the layer expression of Tbr1 has been disrupted. Most noticeably, the difference in expression levels between upper and lower layers is no longer visible. The expression levels have become homogeneous, such that layers that express low levels of Tbr1 on wild-type now also express higher levels in *COUP-TFI*^{flox} mutants. Hence the unlabelled layers IV/V are no longer visible and the strong labeling in layer VI is distributed across the radial surface (Fig. 25, A, A', B, B').

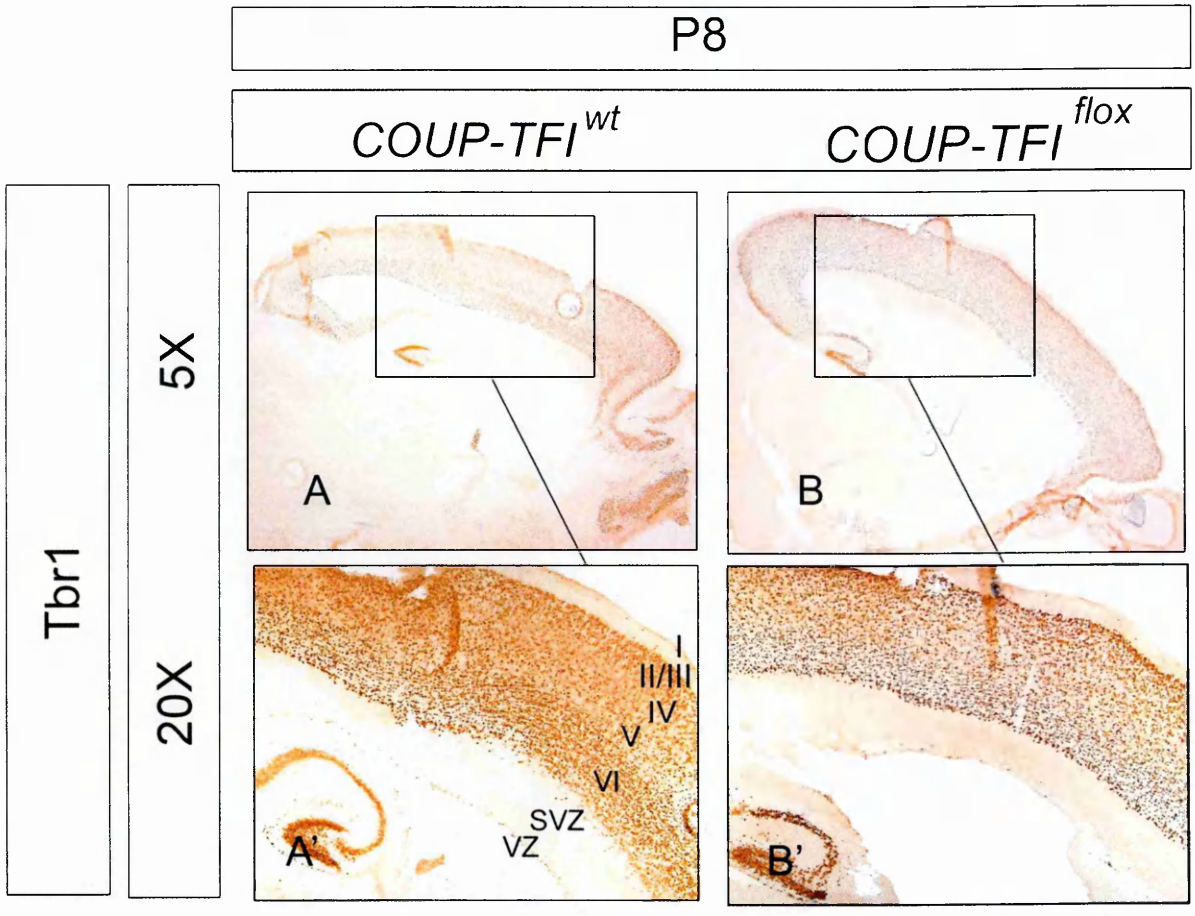


Fig. 25. Tbr1 staining at P8 in wild-type sections labels layers VI strongly as well as upper layers II/III (A'). Layers IV, V are least strongly labeled, particularly at caudal levels (A). *COUP-TFI*^{flox} mutants show a dispersion of layer VI labeling along the radial dimension, such that upper layers are also more strongly labeled (B'), as well as a caudal increase in labeling (B).

The state of the lower layers was also investigated using the marker *Foxp2*, a transcription factor of the Forkhead box superfamily, whose expression is specific to layers V and VI (Ferland et al., 2003). At P8 *Foxp2* is seen expressed strongly in layer VI, which forms a dense, discrete layer, and less strongly in layer V, in which *Foxp2*-positive cells are scattered more sparsely. The two layers of expression appear clearly distinct in the wild-type (Fig. 26, A). However, in the *COUP-TFI*^{flox} mutant, the layers of *Foxp2* expression become continuous and possibly even spread more superficial to layer V (Fig. 26, red arrow in B), suggesting an expansion or a

disorganization of lower layers.

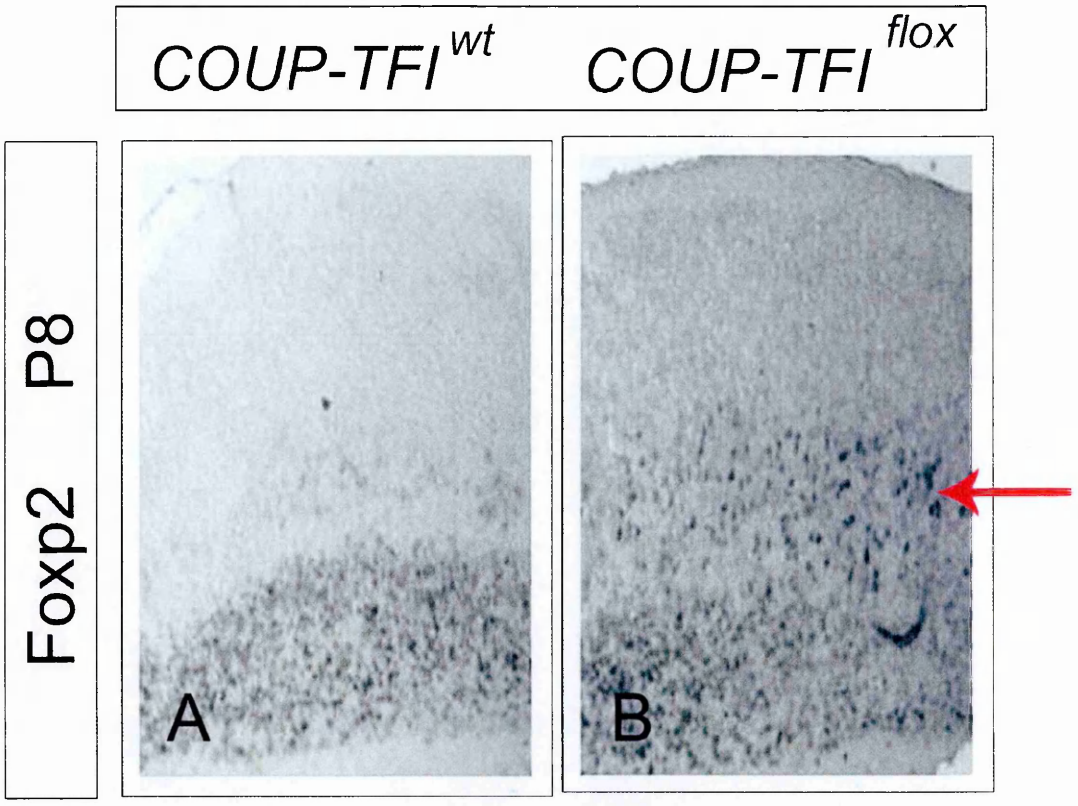


Fig. 26. A: *Foxp2* expression in *COUP-TFI*^{wt} (A) marks layers VI and can be lightly detected in layer V. In *COUP-TFI*^{flox} mouse, expression of *Foxp2* in layers VI and V is spread across both layers (red arrow in B).

A similar phenotype of lower layer spreading is seen using the marker *Fez*, a zinc finger transcription factor specific for layer V corticospinal motor neurons (Molyneaux et al., 2005). *Fez*'s expression pattern is very restricted in wild-type sections (Fig. 27, A). In *COUP-TFI*^{flox} mutants, this expression is expanded into lower layers V and VI, suggesting that these layers are not correctly specified.

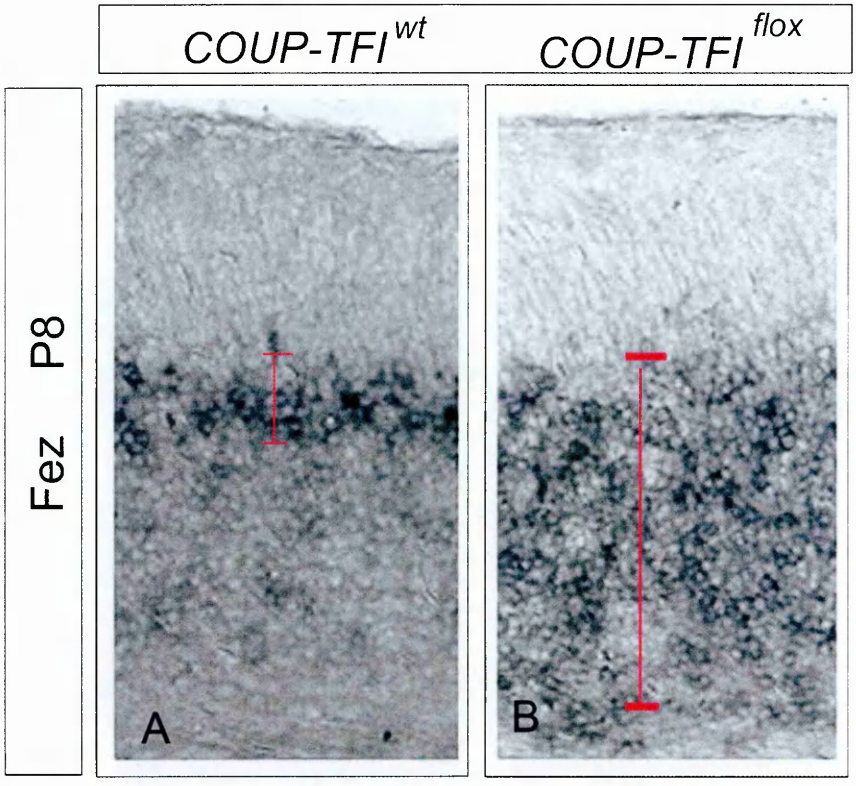


Fig. 27. *Fez* is expressed in upper layer V neurons at P8 in wild-type tissue (A). The domain of expression is expanded into lower layer V and layer VI in *COUP-TFI*^{flox} mutants (red bars in A and B).

We next went on to inspect the formation of upper layers IV and II/III. The disruption of layer IV was previously reported in the null mutant, where it was postulated to be a consequence of the thalamocortical axon pathfinding defect (Zhou et al., 1999). To inspect changes in layer IV, we used the marker *ROR- ß*, a RAR-related nuclear orphan receptor (Nakagawa and O'Leary, 2003) at E16.5, E18.5, and P8. In the wild-type at E16.5 and E18.5 presumptive layer IV cells express high levels of *ROR-ß* mainly in anterior domains, while at P8 *ROR-ß* expression in layer IV is detected posteriorly (Fig. 28, red arrows in C'). In the *COUP-TFI*^{flox} mutants levels of *ROR-ß* expression start to decrease at E16.5, and are completely downregulated from E18.5 onwards (Fig. 28, red arrows in A', B'). These data suggest that COUP-TFI acts intrinsically on layer IV differentiation and that downregulation of *ROR-ß* is a direct

effect of the absence of COUP-TFI in the developing cortex.

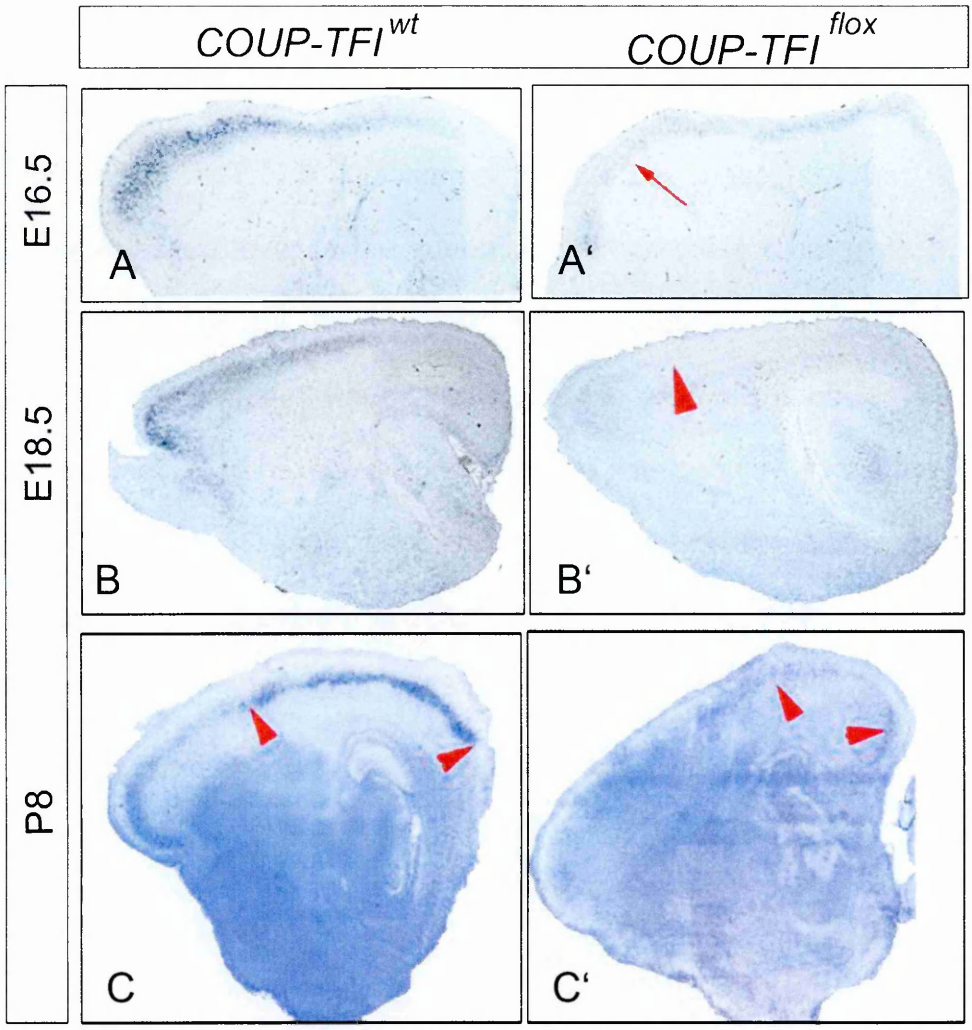


Fig. 28. *RORβ* expression in *COUP-TFI*^{wt} (A-C) and *COUP-TFI*^{flox} (A'-C") at E16.5-P0. The *COUP-TFI*^{floxl} mutant shows nearly uniform low-level plate expression compared to the *COUP-TFI*^{wt}, which shows expression in the intermediate zone at E16.5, followed by expression in layer IV at E18.5 (nascent layer IV) and P8.

A similar phenotype of decreased upper layers is seen using the marker *SCIP*, a POU domain transcription factor expressed in layers V and II/III (Frantz et al., 1994). In wild-type brains at P8, expression of *SCIP* in layers II/III is well defined (Fig. 29, A), while in *COUP-TFI*^{flox} mutant brains, *SCIP* expression is decreased and disorganized

such as a few *SCIP*-positive cells are present in layer IV (Fig. 29, arrows in B).

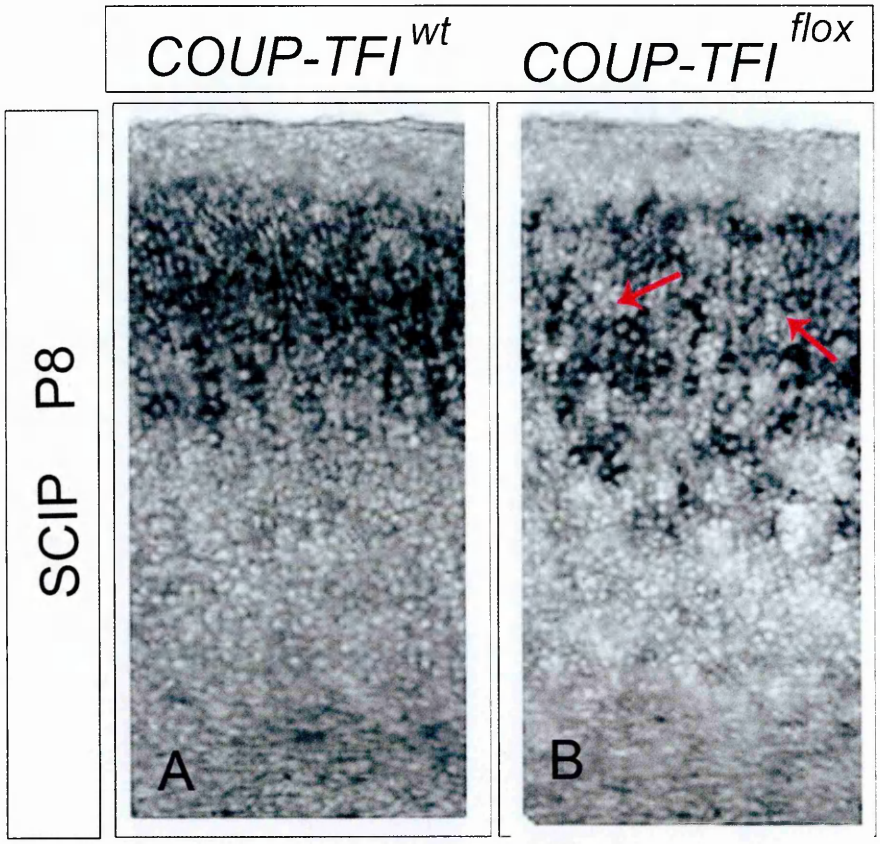


Fig. 29. *SCIP* is expressed in upper layers II/III in wild-type cortex (A), while in *COUP-TFI*^{flox} mutant sections the expression contains gaps and less restricted to the layer II/III domain (red arrows in B).

We then attempted to assess whether these changes in lamination could be related to abnormal cell migration. To do this we carried out BrdU injections at E13.5 and E15.5, time points at which lower and upper layers are being generated respectively, and analyzed the distribution of neurons generated at these time points across the layers in wild-type and *COUP-TFI*^{flox} mice at P0.

The analysis of neurons labeled with BrDU at E13.5 in wild-type mice showed strong labeling of cells in lower layers V and VI, with no labeling in upper layers at P0 (Fig.

30, A). In the *COUP-TFI*^{flox} mutant at P0, the overall number of BrDU-positive cells is not changed (see histogram in Fig. 30), however the distribution of cells along the radial dimension is disrupted, and the labeled cells are not as spatially restricted to lower layers and some labelled cells are also detected in upper layers (Fig. 30, red arrow in B). At E15.5, injection of BrdU labels nascent upper layers visible as a densely packed layer in the superficial part of the cortical plate (Fig. 31, A). In the *COUP-TFI*^{flox} mutant, the number of cells labeled at this time point is significantly decreased, and again, the distribution of labeled cells is not as restricted as in wild-type sections (see histogram in Fig. 31, B). Interestingly, *in situ* hybridization at E15.5 for *Ngn2,* a neurogenic gene expressed in the ventricular zone also showed a decrease in expression in *COUP-TFI*^{flox} mutant. This could suggest that the decreased number of cells seen when BrdU was injected in the *COUP-TFI*^{flox} mutant at E15.5 might result from a depletion of the ventricular zone progenitor pool at this time (Fig. 32, red arrows in A'-C').

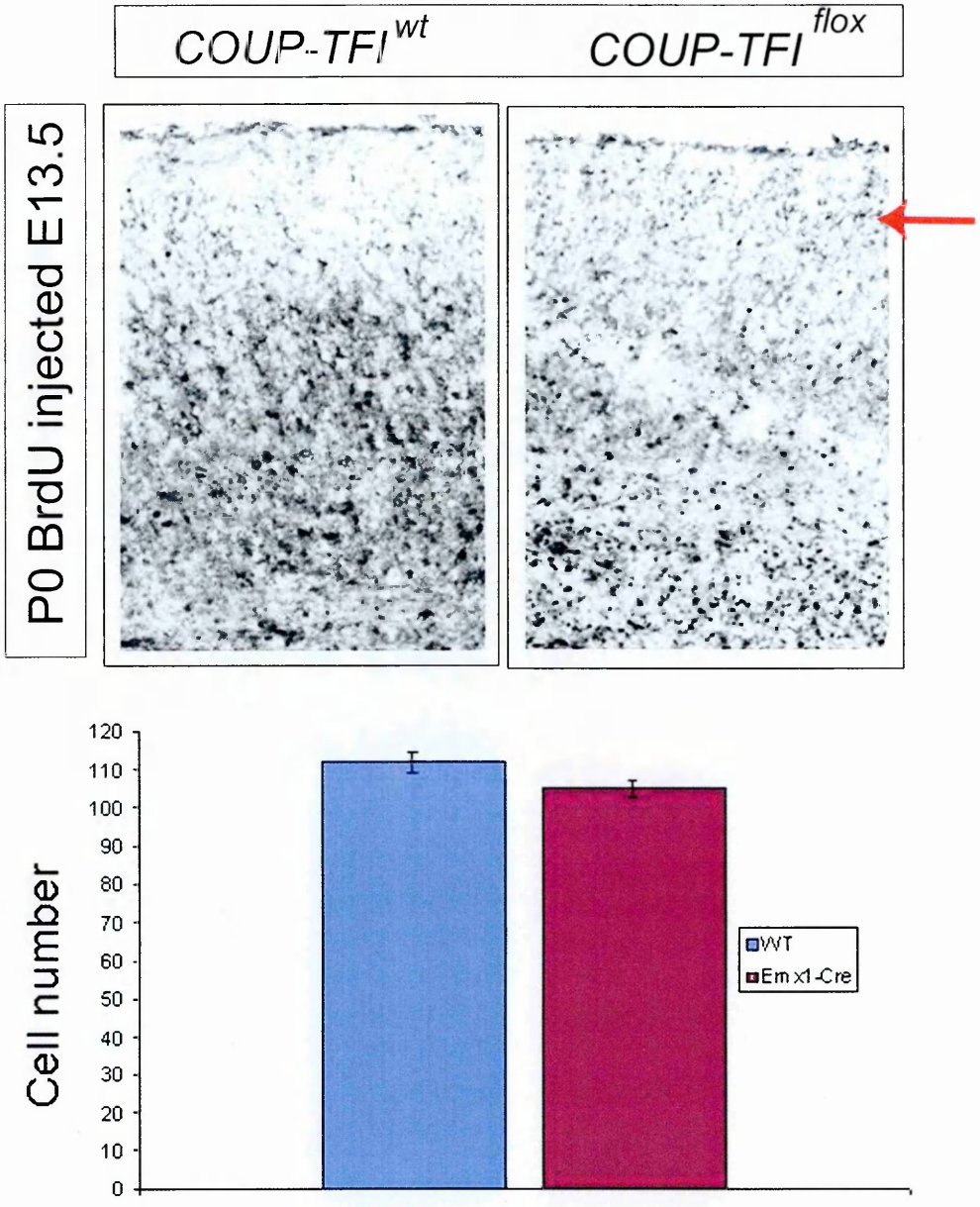


Fig. 30. At P0, BrdU injected at E13.5 labels lower layers in wild-type sections (A), while in *COUP-TFI*^{flox} mutant tissue, the overall number of positive cells is not changed, but their radial distribution is disorganized (arrow in B and histogram).

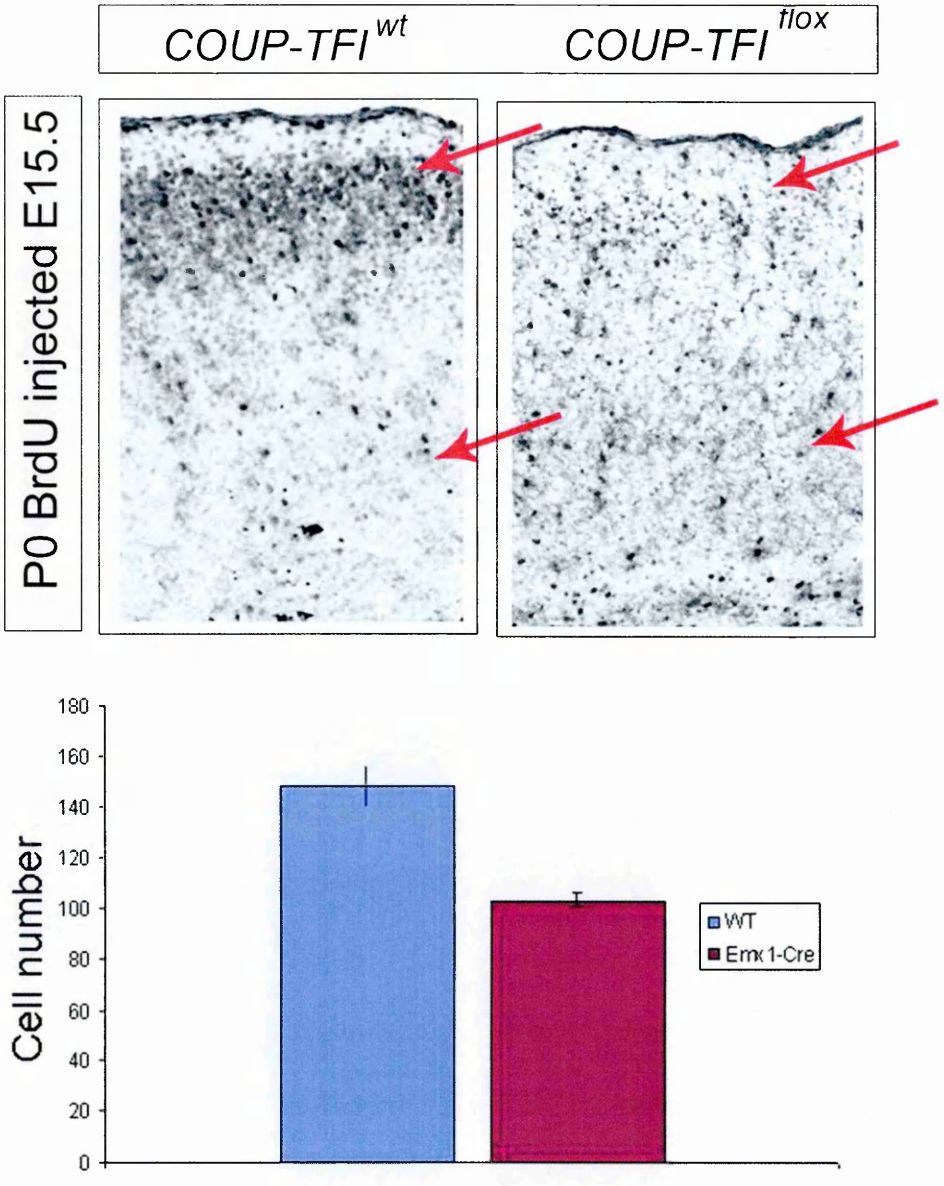


Fig. 31. At P0, BrdU injected at E15.5 labels upper layers in wild-type sections (A), while in *COUP-TFI*^{flox} tissue fewer cells are labeled (B and histogram) and abnormally distributed along the radial dimension (red arrows in B).

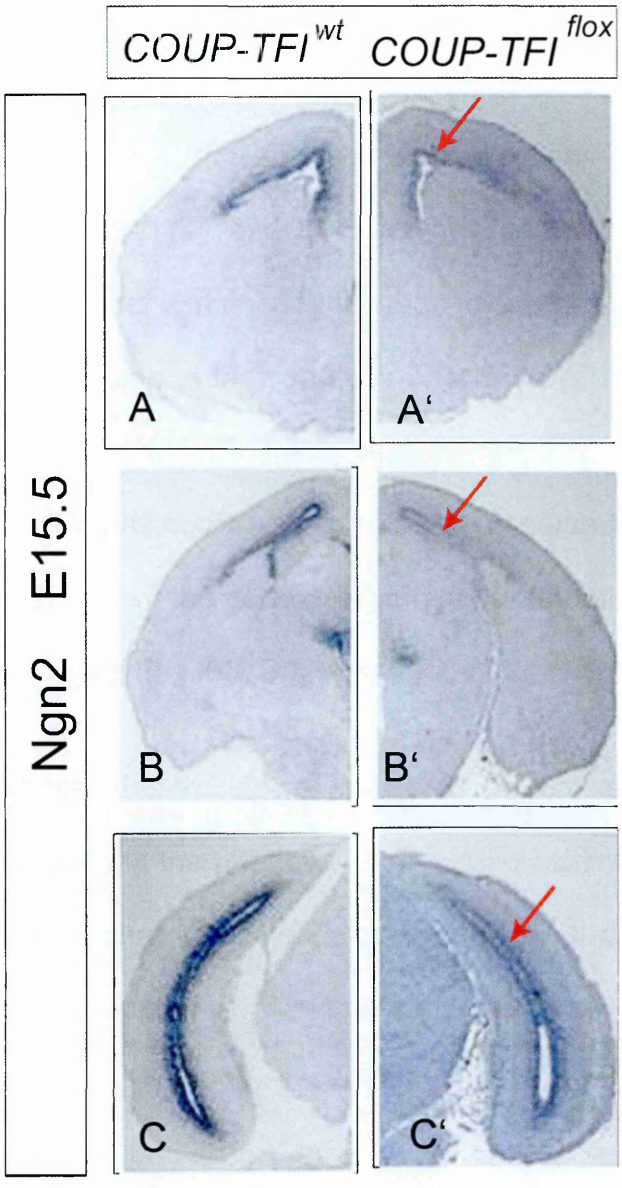


Fig. 32. Expression of Ngn2, a neurogenic gene expressed in the ventricular zone, is decreased in *COUP-TFI*^{flox} mutant tissue at E15.5 (red arrows in A'-C').

In summary, the analysis of the laminar organization in a COUP-TFI conditional mutant revealed defects in all developing layers. In particular, markers for lower layers appeared increased, while markers for upper layers appeared decreased and disorganized. These results were further confirmed by BrdU birthdating analysis, suggesting a problem in the generation and/or migration of cortical neurons.

6. Thalamocortical and Corticofugal Projections

Given that a role for the subplate as an intermediate target for thalamocortical axons before innervating the cortex has been established (Ghosh et al., 1990), and that a previous report on the *COUP-TFI*^{null} mutant suggested that a defect in thalamocortical axon pathfinding had arisen due to a defect in subplate differentiation (Zhou et al., 1999), we investigated whether the *COUP-TFI*^{flox} mutant also displayed a thalamocortical axon pathfinding defect.

To investigate this, lipophlic dye crystals were placed in wild-type and *COUP-TFI*^{flox} mutant developing somatosensory cortices (DiA) and dorsal thalami (DiI) at E15.5. At this stage in the wild-type brain, thalamocortical axons are seen to have left the thalamus (Fig. 33, D), navigated through the basal telencephalon (Fig. 33, C) and internal capsule (Fig. 33, B) and arrive in the cortex, where they navigate along the subplate (Fig. 34, A). However, the axons have not yet innervated their target layer within the cortical plate (Agmon et al., 1993; Miller et al., 1993). These axons can be seen as one bundle traveling through the internal capsule to the cortex (Fig. 33, B and C), where they then start to defasciculate to reach their respective cortical areas. It has been reported that in the *COUP-TFI*^{null} mutant, this pattern of thalamocortical projections is not preserved (Zhou et al., 1999). We investigated the navigation of thalamocortical axons in the *COUP-TFI*^{flox} mutants (Fig. 33, A'-D'). In the *COUP-TFI*^{flox} mutant, no defect such as the one seen in the *COUP-TFI*^{null} mutant was detected. The axons navigate as a single fascicle ventrally from the dorsal thalamus through the basal telencephalon and internal capsule (Fig. 33, red arrow in C', D'), and turn dorsally to enter the cortex (Fig. 34, B).

The placement of a crystal in the somatosensory cortex results in the marking of corticofugal axons, which in the wild-type at E15.5 are seen as a mass of axons in rostral cortex that caudally fasciculate tightly and grow ventrally out of the cortex, into the subpallium and through the internal capsule. Around the internal capsule the close interactions of thalamocortical and corticofugal axons can be seen by the overlapping of the two dyes (Fig. 33, B, C and B', C'). From the internal capsule, the axons navigate medially and dorsally into the thalamus (Fig. 33, D). This pattern of corticothalamic axons is mostly preserved in the *COUP-TFI*^{flox} mutant. The axons grow out of the cortex and into the internal capsule, and arrive in the thalamus, as in wild-type (Fig. 33, D').

These dye-tracing experiments reveal a number of intriguing results. Firstly, comparing the phenotypes of corticothalamic and thalamocortical axons at this stage shows that COUP-TFI expression in the cortex is not required for intact early pathfinding of corticofugal or thalamocortical axons. Comparing *COUP-TFI*^{flox} and *COUP-TFI*^{null} mutant phenotypes of thalamocortical axons allows us to conclude that the subplate defect is not the cause of the thalamocortical defect, as *both COUP-TFI*^{flox} and *COUP-TFI*^{null} mutants have a diminished subplate, but only *COUP-TFI*^{null} mutants have defective thalamocortical axons (Zhou et al., 1999 and M. Armentano, personal communication). Finally, the *COUP-TFI*^{flox} - *COUP-TFI*^{null} comparison also allows us to conclude that the thalamocortical axon pathfinding defect in the *COUP-TFI*^{null} mutant does not arise due to the absence of the protein in the cortex, but must be due to a requirement for COUP-TFI in either thalamus or basal telencephalon for intact pathfinding.

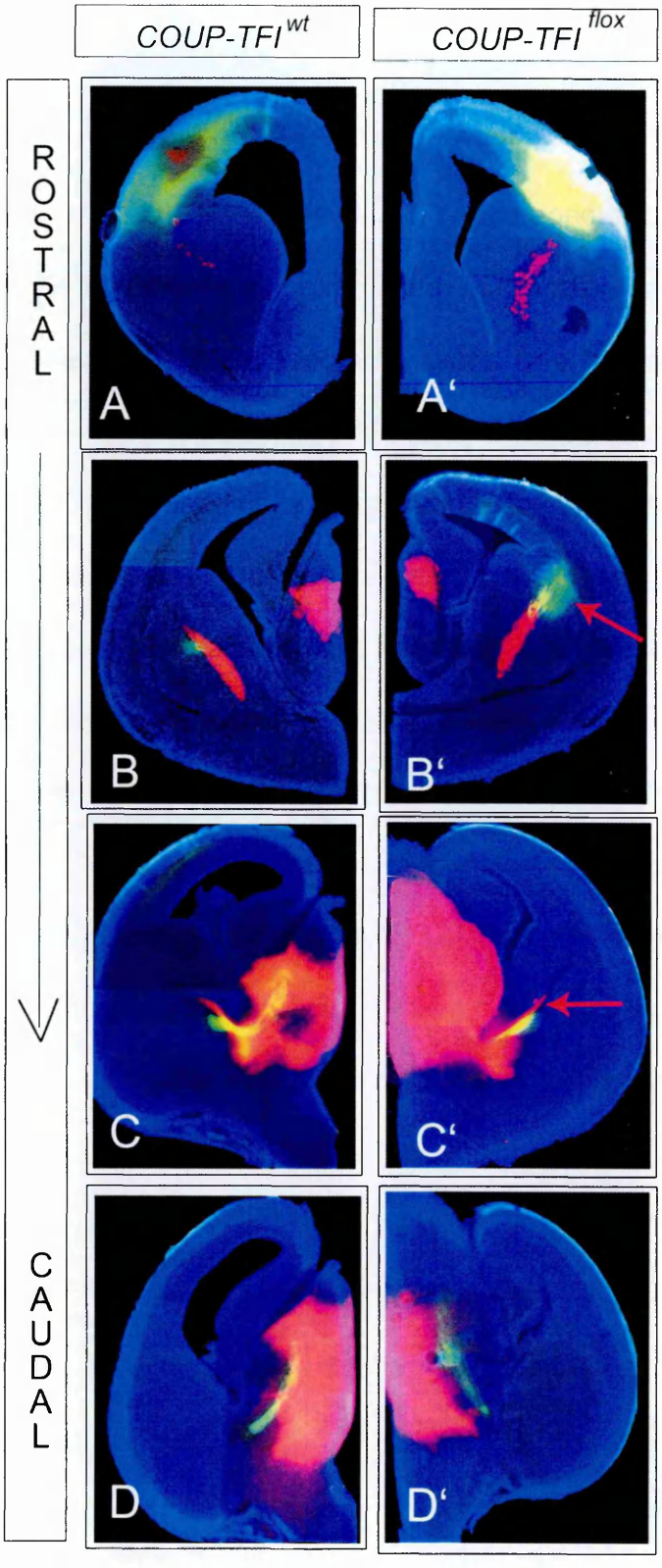


Fig. 33. DiI (red) and DiA (green) tracing of thalamocortical and corticofugal axons, respectively in coronal thick sections from rostral to caudal levels. Thalamocortical axons can be seen to navigate from thalamus to internal capsule in both wild-type and *COUP-TFI*^{flox} mutant sections (A-D'). Corticofugal axons are seen to enter the internal capsule and the thalamus in wild-type and *COUP-TFI*^{flox} mutants (A-D').

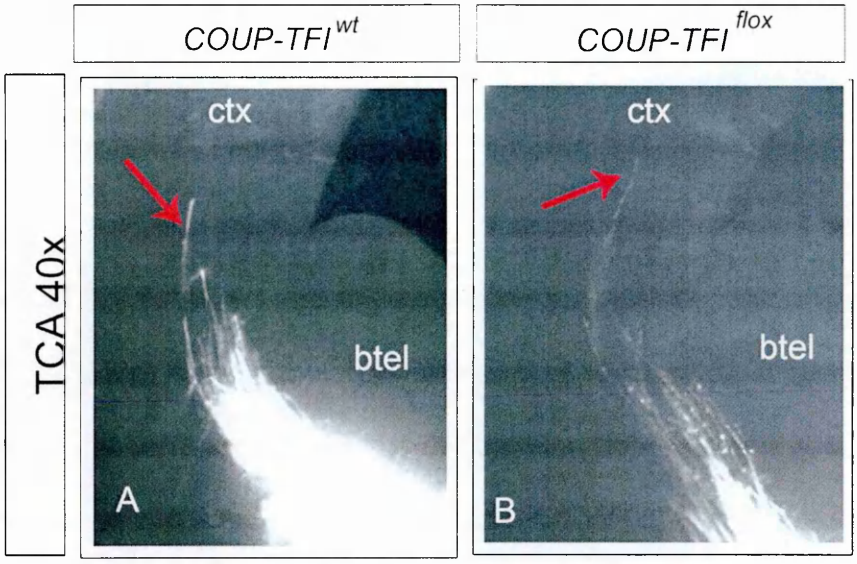


Fig. 34. DiI tracing of thalamocortical axons in wild-type and *COUP-TFI*^{flox} mutant sections at high magnification showing entry of axons into the pallium after exiting the internal capsule (arrows in A, B). No difference was detectable between wild-type and *COUP-TFI*^{flox} mutants. ctx:cortex, btel: basal telencephalon.

III. DISCUSSION

COUP-TFI is an orphan nuclear receptor transcription factor that has previously been implicated in neurogenesis, neuronal differentiation, axon pathfinding and cortical development *in vitro* and *in vivo* (Adam et al., 2000; Studer et al., 2005; Zhou et al., 1999, 2001). The characterization of the COUP-TFI null mutant (Zhou et al., 1999) indisputably established a role for COUP-TFI in corticogenesis, but simultaneously it threw up many new questions and did not provide satisfactory answers to some of the questions it attempted to answer. The global loss of COUP-TFI meant that the primary effects of gene loss, that might be autonomous to one brain region, could not be differentiated from secondary effects in a different brain region. The report concluded that defects in subplate differentiation resulted in a thalamocortical pathfinding defect, and that this consequentially resulted in death of layer IV neurons. However, these conclusions were made without evidence of cell death and without considering that the distance between subplate and the site of the pathfinding defect, the internal capsule, is likely to be too large for secreted guidance molecules to act. This suggested that COUP-TFI may have independent developmental roles in both cortex and thalamus. This hypothesis motivated the making and characterization of the conditional mutant of COUP-TFI, and its crossing with the *Emx1-CRE* line to eliminate COUP-TFI specifically in the cortex.

Subplate and Preplate Development

Using the *COUP-TFI*^{flox} mutant a number of intriguing observations were made that shed light onto the role of COUP-TFI in cortical development. We first investigated the state of the subplate, as it was pinpointed as being the primary cause of defects in thalamocortical axons and layer formation in the *COUP-TFI*^{null} mutant. Our analysis

suggested a decrease in neuronal differentiation during early neurogenesis, resulting in a thinner subplate. The decreased subplate was observed in both *COUP-TFI*^{flox} and *COUP-TFI*^{null} mutants, suggesting that there is an intrinsic requirement in the cortex for COUP-TFI to orchestrate intact subplate formation. Unlike previous reports, we could not replicate that the subplate forms correctly and then degenerated (Zhou et al., 1999). As the subplate forms from splitting of the preplate, the defect seen in the subplate motivated us to inspect development of the preplate. The preplate, like the subplate, appeared decreased in both *COUP-TFI*^{null} and *COUP-TFI*^{flox} mutants, suggesting that the subplate defect might be due to a diminished number of preplate neurons born during early neurogenesis. This further supports the hypothesis that COUP-TFI is intrinsically required for regulating early cortical neurogenesis and that the subplate defect originates in impaired formation rather than maintenance.

At later stages, during cortical plate formation, the absence of COUP-TFI resulted in layer identity being disrupted, such that lower layers appeared increased, while upper layers appeared decreased. During late cortical development we also observed more intermediate progenitors in the subventricular zone and decreased apoptosis. The defects in neurogenesis and differentiation were all consistent between *COUP-TFI*^{null} mutant and *COUP-TFI*^{flox} mutant, while a thalamocortical axon pathfinding defect was found to be specific to the *COUP-TFI*^{null} I mutant.

Axon Pathfinding in *COUP-TFI*^{flox} and *COUP-TFI*^{null} Mutants.

The subplate acts as an intermediate target for thalamocortical axons navigating to their cortical target layer (Ghosh et al., 1990) The fact that in the *COUP-TFI*^{null} mutant, the subplate is diminished and thalamocortical axons display a pathfinding defect before reaching the cortex, lead Tsai and colleagues to conclude that the pathfinding

defect was a consequence of the loss of guidance cues from the subplate that are instrumental in guiding thalamocortical axons. If this were the case, we would expect a thalamocortical axon defect in both the null and *COUP-TFI*^{flox} mutants, as they show similar subplate defects. However, we were also aware that the thalamocortical pathfinding defect might be due to a requirement for COUP-TFI outside of the cortex, motivating us to carry out axonal tracing experiments in the *COUP-TFI*^{flox} mouse. These revealed that at E15.5, the *COUP-TFI*^{flox} mutant shows intact thalamocortical and corticofugal projections. At this stage, the *COUP-TFI*^{null} mutant already shows a defect at the level of the internal capsule (Zhou et al., 1999 and M. Armentano, personal communication), suggesting that there is a requirement for COUP-TFI outside of the cortex that regulates thalamocortical axon pathfinding. It is plausible that the lamination defects described above result in an inability of axons to innervate their correct target layer at later stages. It has previously been reported that corticothalamic axons arising from the visual cortex in the null mutant projected axons to the ventrobasal thalamus rather than the lateral geniculate nucleus (Zhou et al., 2001). It will be interesting to see whether future DiI labeling in the *COUP-TFI*^{flox} mutant at later developmental stages will reveal a similar defect in corticothalamic pathfinding. Such a defect would be concordant with the changes in lamination and rostrocaudal patterning discussed below.

Hence the comparison between *COUP-TFI*^{flox} and null mutant phenotypes of thalamocortical axons allows us to conclude that the subplate defect is not the cause of the thalamocortical defect, as both *COUP-TFI*^{flox} and *COUP-TFI*^{null} mutants have a diminished subplate, but only null mutants have defective thalamocortical axons. Furthermore, the *COUP-TFI*^{flox} - *COUP-TFI*^{null} comparison allows us to conclude that

the there must be a requirement for COUP-TFI in either thalamus or basal telencephalon for intact thalamocortical pathfinding.

COUP-TFI in Development of Cortical Layers

Thalamocortical axons have been suggested to impart area identity to developing cortical plate (Schlaggar and O'Leary, 1991), as well as being able to provide trophic support to different cell types at different time points during development (Price and Lotto, 1996; Gitton et al., 1999). During prenatal development, it has been suggested that thalamocortical axons provide trophic support for subplate cells, which are partially eliminated once thalamic axons enter the cortical plate (Price and Lotto, 1996). During postnatal development, thalamic innervation has been suggested to be required for the correct differentiation or maintenance of layer IV, the target of thalamocortical axons. This has been proposed due to the lack of expression of a layer IV-specific LacZ maker, H-2Z1, in which expression is dependent upon thalamocortical innervation after birth (Gitton et al., 1999). Furthermore, the requirement for thalamocortical innervation for intact cortical plate development has been suggested by Tsai and colleagues. This was based on the COUP-TFI null mutant's thalamocortical axon defect, which was proposed to be the cause of a loss of layer IV neurons (Zhou et al., 1999). If cortical plate development depended on an intact thalamocortical axon projection, the *COUP-TFI*^{flox} mutant would not display defects during later cortical development, while the null mutant would. This would specifically be the case if during cortical development, external patterning mechanisms, such as thalamocortical axons, were more important than intrinsic factors, such as appropriate gene expression. If cortical plate development is primarily intrinsically regulated, and independent of thalamocortical innervation, one

would expect that the *COUP-TFI*^{flox} mutant will display defects that arise due to the absence of COUP-TFI.

This hypothesis was confirmed by the use of a number of layer-specific markers once the cortical plate had developed. Many of these showed that compared to wild-type, *COUP-TFI*^{flox} mice have an increased spread of markers for lower layers (e.g. *Fez, Foxp2* in layers V, VI), but a decrease of markers of upper layers (e.g. RORb, SCIP in layers IV, II/III). Interestingly, Tbr1, which labels mature neurons, also showed a homogeneous labeling of upper and lower layers rather than the strong expression, which is restricted to layer VI in wild-type.

As many layer-specific markers are also expressed in an area-specific pattern, some of these experiments could be used to make conclusions about the role of COUP-TFI in arealization. Tbr1 and *Foxp2* for example, are expressed differentially between different brain areas. In the adult, Tbr1 is expressed more strongly in all layers rostrally, while caudally its expression is limited to layers VI and II/III. The expression pattern of Tbr1 seen in the *COUP-TFI*^{flox} mutant resembles that seen in the wild-type rostral cortex, indicating that caudal areas have adopted rostral characteristics in the absence of COUP-TFI. Hence the homogeneity of Tbr1 expression across layers in the *COUP-TFI*^{flox} mice was combined with a change in expression that suggested a defect in arealization. A role for COUP-TFI has previously been suggested in cortical arealization by Tsai and colleagues, who showed changes in region-specific markers such as *Id2, Cad8 and ROR-ß* (Zhou et al., 2001). The combination of the putative changes in area identity and layer identity seen here result in a cortex that has more rostral characteristics and an increase in lower layers. Wild-type rostral cortex, compared to caudal cortex, is in fact characterized by thicker lower layers, which

send axons to subcortical areas such as motor neuron projections to spinal cord, and thinner layer IV, which receives inputs from subcortical layers, such as sensory projections from the thalamus. The synthesis of these phenotypic changes might result in an enlargement of areas that have motor cortex-like properties, and a decrease in areas that have somatosensory and visual cortex-like properties.

These results, tied together with the DiI experiments described above, are informative about the role of thalamic innervation in cortical pattering. The interdependence of cortical patterning and thalamic axons has been the subject of longstanding debate. Early transplantation studies suggested that the cortex might be homogeneous and undetermined in its area identity before the arrival of thalamocortical axons (Schlaggar and O'Leary, 1991). However, more recently, analysis of genetic experiments has suggested that the cortex contains intrinsic mechanisms of patterning that are independent of thalamocortical axons (Miyashita-Lin et al., 1999; Nakagawa et al., 1999). Here we provide further support to the mounting evidence for the latter hypothesis. Furthermore, we give evidence that intact patterning of the cortex is in turn not required for the navigation of thalamic axons to the cortex. It is plausible that within the cortex, at later stages, the defect in cortical patterning could give rise to thalamic axons not targeting the correct subcortical area.

While a role for COUP-TFI in cortical arealization has previously been described in the null mutant, the only lamination defect identified was the loss of layer IV. The description of the development of defects in the cortical plate from E15.5 to P8 is a novel contribution to our understanding of the functions of COUP-TFI in vivo. Together with the defects in arealization, these results emphasize how laminar and area development are two closely linked processes that are regulated in concert. Finally, the fact that *COUP-TFI*^{null} and *COUP-TFI*^{flox} mutants had similar phenotypes

where null tissue was available, suggests that regulation of arealization and lamination is intrinsic to the cortex.

COUP-TFI in Neurogenesis

Many of the defects reported here in subplate, preplate and cortical layer formation are likely to result either from neurons not differentiating properly, or not migrating to their target correctly. To investigate these possibilities, we used BrdU injections and markers of ventricular and subventricular zone progenitors. These markers showed that at early stages the ventricular zone and intermediate progenitors appeared increased. Together with the decreased preplate and subplate, these data suggest that during early neurogenesis, COUP-TFI is acting as a positive regulator of differentiation. This would be concordant with biochemical studies showing that COUP-TFI acts as a positive regulator of neuron-specific genes (Chew et al., 1999; Fernandez-Rachubinski et al., 2001). However, the majority of previous studies on the role of COUP-TFI in neurogenesis in vitro, has suggested that COUP-TFI acts as a suppressor of neuronal differentiation and a repressor of genes up-regulated by retinoic acid (Adam et al., 2000; Studer et al., 2005; Cooney et al., 1992).

As cortical development progresses, the *COUP-TFI*^{flox} mutant displays a phenotype which could result from the role for COUP-TFI as an inhibitor of neuronal differentiation. At E15.5, we see a decrease in expression of *Ngn2*, a marker of a ventricular zone, and once the cortical plate is fully developed, we see an increase in markers of lower layers as well as a decrease in markers of upper layers. This could result if divisions, which in the wild-type mice give rise to upper layer neurons, in the *COUP-TFI*^{flox} mice give rise to neurons with lower layer identity instead and hence deplete the progenitor pool prematurely. It is hence possible that the role of COUP-

TFI in regulating the balance between proliferation and differentiation differs during the genesis of the preplate and the genesis of cortical plate neurons.

Analyzing new and existing expression data for COUP-TFI has given us a hint for a possible change in the requirement for COUP-TFI over time in different parts of the cortex. While immunofluorescence indicates that COUP-TFI protein is present in the most cells within the cortex, it is also apparent that the expression levels are higher within the ventricular zone than in differentiated neurons (Fig. 8). Inspecting mRNA expression from rat E12-P7 (Liu et al., 2000) shows that COUP-TFI message is present in the ventricular zone until E13.5, and subsequesntly starts to be expressed in cortical plate neurons as well. Double immunostaining and *in situ* hybridization for *COUP-TFI* mRNA and protein at E13.5, when the cortical plate starts to form, shows that the expression of *COUP-TFI* mRNA is limited to the ventricular zone at E13.5, while the protein can be detected in both the ventricular zone and preplate (Fig. 35). Prior to this, in preplate and subplate neurons, although no mRNA is seen, low levels of COUP-TFI protein can be detected in the cells. This suggests that in early-born neurons produrance of COUP-TFI results in the presence of the protein, but not the transcript. One can hence speculate that the primary requirement for COUP-TFI is in the ventricular zone at this time, and not in the neurons of the preplate and subplate. Once the cortical plate is generated, however, both *COUP-TFI* mRNA and protein expression are seen within cortical plate neurons. This timing coincides with the birth of layers. This differential expression of *COUP-TFI* mRNA over time may account for why some layers show more pronounced deficits compared to the preplate and subplate. Furthermore, the neurons of the cortical plate differ from those of the preplate and subplate in that they represent cells that persist throughout adulthood.

Both the preplate and subplate consist of early neuronal populations that play important roles during development, but many of which die before birth.

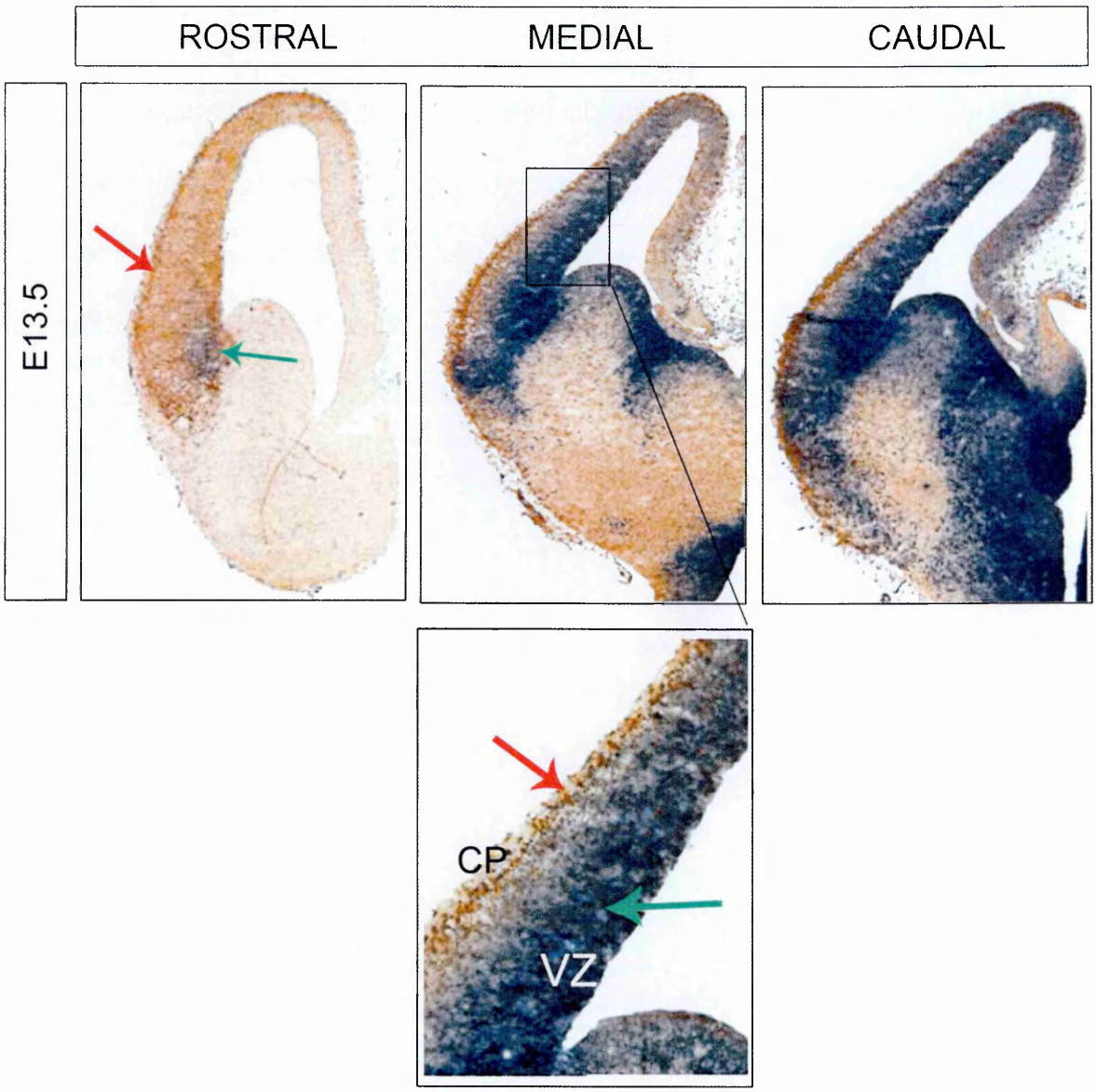


Fig. 35. COUP-TFI immunostaining (DAB, brown) and *in situ* hybridization (Digoxigenin, blue) on wild-type sections at E13.5. COUP-TFI protein is detected in both the ventricular zone and cortical plate (red arrows), while *COUP-TFI* mRNA is only detected in the ventricular zone at this stage.

In the adult cortex, *COUP-TFI* mRNA is seen in a layer and area-specific fashion with high caudal and lateral expression and high expression in upper layers IV and II/III and low expression in deep layers V/VI. Not surprisingly, wild-type COUP-TFI is expressed in the layers which are most diminished in the *COUP-TFI*^{flox} mutant, suggesting that its expression is required for the identity of late-born neurons of upper layers. These expression patterns indicate that *COUP-TFI* is differentially regulated over development and this differential regulation could potentially be linked to functional changes. Previous understanding of the role of COUP-TFI in neurogenesis had been limited to *in vitro* assays. These experiments described here reveal a novel requirement for COUP-TFI in regulating neurogenesis and neuronal differentiation that had not previously been described *in vivo* in mouse.

In *Drosophila,* the homolog of COUP-TFI, Sevenup (Svp) has been recently described as a regulator of a switch between early and late-born neuronal fates. Doe and colleagues have carried out comprehensive cell lineage studies to identify the clone of neurons and glia produced by all 30 embryonic neuroblasts and have characterized the birth-order of all progeny for selected neuroblasts (e.g Doe, 1992). By doing this, they have identified that each neuroblast expresses a cascade of transcription factors (Hunchback, Krüppel, PDM and Castor) that is progressed through in a conserved temporal order (Fig. 36). While neuroblasts progress through this cascade, the daughter ganglion mother cells maintain the expression of the transcription factor that was expressed at its birthdate (Isshiki et al., 2001). Hence, in the progression from expression of Hunchback, Krüppel, PDM and Castor found in neuroblasts, early-born ganglion mother cells and neurons express Hunchback, while second-born neurons express Krüppel.

Hunchback is necessary and sufficient for an early-born fate, and its overexpression in neuroblasts has the potential to restart the lineage, indicating that there is some degree of plasticity in the developmental potential of the progenitors (Pearson and Doe, 2003). Similar to the plasticity seen in cortical progenitors upon transplantation, the ability to adopt early-born fates decreases with the age of the progenitor (Pearson and Doe, 2003; Desai and McConnell, 2000). In the progression through this cascade described in ganglion mother cells, Svp is expressed transiently as the neuroblast switches from expression of one temporal marker to another. The loss of Svp expression hence resulted in an inability of progenitors to switch fate, and an overproduction of Hunchback-positive neurons in many neuroblast lineages. Overexpression of Svp, on the other hand, resulted in a loss of early-born neuron (Kanai et al., 2005). The expression of *svp* mRNA is seen in neuroblasts, while the protein is expressed exclusively in neurons and regulation of expression has been found to be dependent on the protein Prospero, which is asymmetrically localized to ganglion mother cells, and on the event of mitosis (Mettler et al., 2006). Hence the transient expression of Svp mediates the establishment of neuronal diversity.

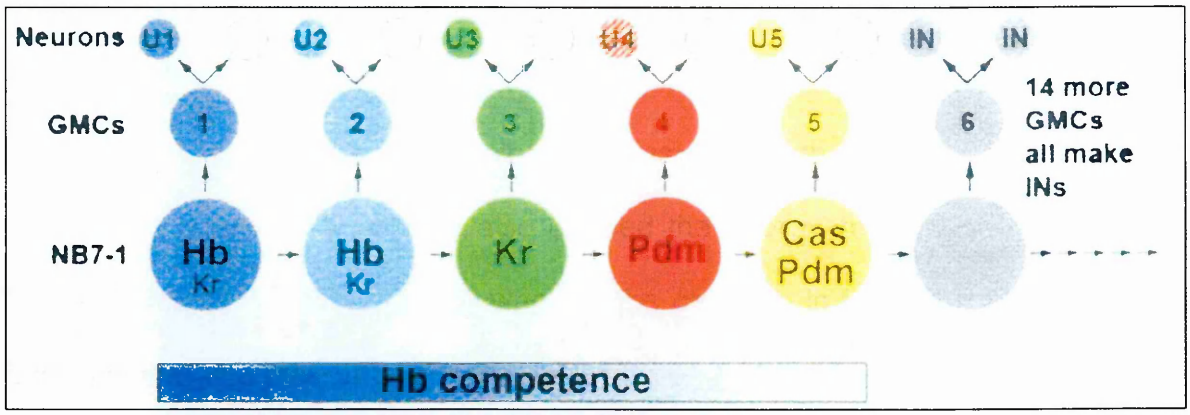


Fig. 36. *Drosophila* neuroblasts (here NB7-1) express a cascade of transcription factors (Hunchback, Krüppel, PDM and Castor). The expression of these transcription factors is transient in neuroblasts, but maintained in ganglion mother cells (GMCs) and neurons (e.g. U1, U2 etc.), where their expression can be considered markers of temporal identity of a neuron's birthdate. The competence of a neuroblast to respond to Hunchback expression decreases with time. Taken from Cleary and Doe, 2006.

This scenario resembles cortical development and the role of COUP-TFI in a number of ways. Like in *Drosophila*, the cortex is characterized by the expression of transcription factors that characterize the birthdate of neurons. These are the layer-specific markers, such as *ROR-ß* or *Fez*, which act in concert with area-specific markers to establish neuronal identity. If one were to consider lower layers early-born neurons, parallel those expressing Hunchback, and upper layers late-born neurons, parallel to those expressing PDM and Castor, it is striking how the loss of COUP-TFI and Svp results in similar phenotypes of an overproduction of early-born neurons, and a decreased production of late-born neurons, as though a switch in fate were not taking place. The differential spatiotemporal regulation of *svp* and *COUP-TFI* mRNA and protein expression is a further hint that COUP-TFI may be regulating neuronal identity in a fashion similar to that of Svp. However, it is apparent that the complexity of the mammalian cortex is such that the number

of divisions and neuronal identities outnumbers those seen in the fly, and it is likely that this complexity is in part accounted for by the multifunctionality of genes such as COUP-TFI. Hence while COUP-TFI may be regulating a switch between upper and lower layer neuronal fate, it may also be involved in the progression from intermediate progenitor production to preplate neurons.

A number of lines of current research in mammalian development suggest that, beyond COUP-TFI, the orchestration of cortical development may be largely regulated by intrinsic determinants acting in a fashion similar to Hunchback and Krüppel. This has been suggested both by *in vitro* studies showing that cortical progenitors have an intrinsic capacity to produce neurons expressing markers of temporal and layer identity in the correct sequence in culture, as well as by *in vivo* studies identifying individual genes, such as *Foxg1* and *Fezl* as intrinsic determinants of specific temporal and laminar fates (Shen et al., 2006; Hanashima et al., 2005; Chen et al., 2005).

Concluding Remarks and Future Directions

In this report we described the use of a conditional mutant approach to investigate the role of COUP-TFI during cortical development. We have provided preliminary evidence for a novel role of COUP-TFI as a pro-differentiation factor in early cortical neurogenesis and as a regulator of neuronal diversity during the formation of the cortical plate. COUP-TFI appears to act as a regulator of upper versus lower layer neuronal cell fate in a way that is reminiscent of the function of its fly homolog, sevenup, in regulating early versus late neuronal fate in the eye and central nervous system. The elimination of COUP-TFI from the cortex specifically also allowed us to

identify its role outside of the cortex in guiding thalamocortical axons through the internal capsule.

While these data have provided interesting insight into the role of COUP-TFI during cortical development, they have also opened the door to many further questions. It will be of interest to characterize whether the changes in laminar identity described here result in changes of layer-specific projection of lower and upper layers, such as to the spinal cord and the corpus callosum. Furthermore, if COUP-TFI acts as a regulator of laminar fate, its overexpression should be sufficient to change the fate of neurons to an upper layer fate. One could test this hypothesis by viral transfer of *COUP-TFI* cDNA during early stages of neurogenesis, when lower layers should be generated, and characterizing whether this time-specific overexpression results in an adoption of upper layer characteristics. Given that some targets of COUP-TFI have recently been identified (M. Armentano and M. Studer, personal communication), it would be interesting to see whether there is an effector of COUP-TFI that could orchestrate rescue of the phenotype we have characterized. Finally, while we have gained insight into the role of COUP-TFI in neurogenesis by removing it only from the cortex, the removal of COUP-TFI in individual clones within the cortex genetically or by transplantation would allow us to characterize how the divisions of progenitors differ in the absence of COUP-TFI in a cell-autonomous manner. The current data provides exciting prospects for further insights into the programs regulating the equilibrium between proliferation and differentiation and the generation of neuronal diversity in the neocortex.

IV. MATERIALS AND METHODS

Gene Targeting

COUP-TFI^{nul} and *COUP-TFI*^{flox} mice were generated by Maria Armentano in Dr. M. Studer's laboratory using a gene targeting vector in which two lox sites flank exon three and the polyadenylation site of COUP-TFI, and a third lox site is located downstream of the selectable neomycin (neo) resistance gene (see also Fig. 4 in Results section 1). This vector contained 4kb of 5' and 1.4kb of 3' homologous genomic sequences and a diphteria toxin fragment A (DTA) cassette for negative selection (kind gift of P. Soriano). The targeting vector was electroporated into TBV2 ES cells according to standard protocols and homologous recombination events were identified by Southern blotting. To obtain *null* and *flox* alleles, the *COUP-TFI flox/neo* positive clone was electroporated with a plasmid coding for the *Cre-recombinase*. For the null allele, clones in which the third exon and the neo gene were excised were screened by PCR and identified as *COUP-TFI null* clones. For *flox* alleles, clones in which the neo gene only was excised were screened for and identified as *COUP-TFI flox* clones. These clones were subsequently injected into C57BL/6 blastocysts and the resulting chimeras were then mated to C57BL/6 females to obtain germline transmission. All studies were carried out in a mixed C57BL/6-129Sv genetic background. All experiments were conducted in accordance with guidelines of the Institutional Animal Care and Use Committee, Cardarelli Hospital, Naples, Italy.

Genotyping and Tissue Procurement

Gravid dams were sacrificed by cervical dislocation around midday of the required stage, and embryos removed from uterine sacs and stored on ice in phosphate-buffered saline (PBS). Brains were dissected out of crania in PBS and numbered corresponding to tail tissue pieces. Brains were incubated overnight at 4°C in 4% PFA in PBS, and then passed through 10%, 20% and 30% sucrose gradient for cryoprotection before including brains in OCT and crytostat sectioning.

Genomic DNA was extracted from tail tissue fragments by incubation overnight in 300μl tail lysis solution with 1μg /μl proteinase K at 55°C. After incubation, 900 μl cold 96% ethanol was added to Eppendorf tubes, and these were shaken to form an aggregate, which was then precipitated by centrifugation (10mins, 13krpm). Supernatant was removed and 500μl of cold 70% ethanol was added and centrifuged (5mins, 13krpm). Supernatant was removed, pellets air-dried and re-suspended in 500μl of H_2O milliQ by heat and agitation.

Genotyping of embryos was carried out by polymerase chain reaction for *COUP-TFI null, floxed* and *CRE* loci (Fig. 37). The wild-type and the *null* loci were detected by use of three primers (1: forward primer 5' of exon3: CTGCTGTAGGAATCCTGTCTC; 2: backward primer within exon3: AATCCTCCTCGGTGAGAGTGG; 3: backward primer 3' of exon3: ACATACACAGCCTGGCCT), such that two possible fragments could be amplified. In the wild-type, a band of 240bp was amplified between primers 1 and 2, while in the null, a fragment of 270bp was amplified by primers 1 and 3 (Fig. 38).

The wild-type and floxed conditional loci were detected by two primers (1: forward primer 5' of exon3, see above; 2: backward primer within exon3, see above). The presence of the *Cre*-recombinase was detected by two primers (4: forward primer:

CAACCGAGACCTTCCTGTTC; 5: backward primer: ATGTGGATGGAGAGAGGTCG). PCRs for the *Cre* gene was accompanied by a control PCR, which is an ubiquitously expressed gene (Fig. 38).

All reactions were carried out using the following program in a thermocycler:

Step 1: 10 mins 95°C

Step 2: 1 min 95°C

Step 3: 1 min 60°C

Step 4: 1 min 72°C, repeat steps 2-4, 35x

Step 5: 10min 72°C, then keep at 4°C until loading gel.

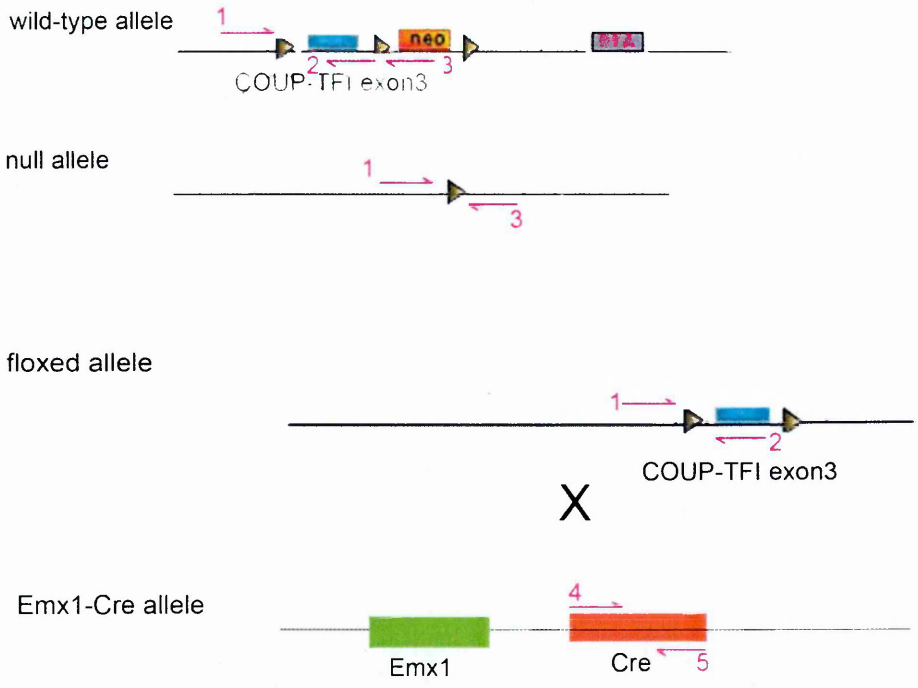


Fig. 37. Schematic of PCR strategy. Null mutants were identified by amplification of sequences in exon three by one forwards and two reverse primers. The putative band produced by primer 3 and 1 is too long for amplification resulting in a single product between 1 and 2 in wild-type and a single product between 1 and 3 in null mutants. Primers 1 and 2 were used to detect lox sites in COUP-TFI^{flox} animals by the difference in product length in the presence of the lox locus (~50bp). The Cre allele was detected by amplification of sequences within Cre.

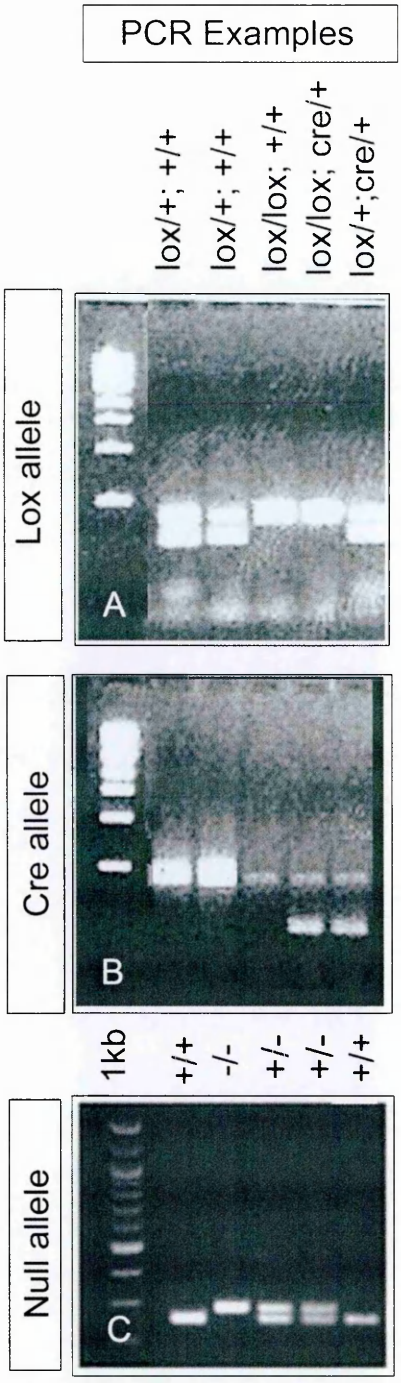


Fig. 38. Genotyping was carried out by PCR amplification of *Lox* and *Cre* alleles (A, B) for identification of conditional mutants and of sequences in exon three for identification of null mutants (C).

Immunohistochemistry and Antibodies

Brains were cryosectioned according to standard procedures. Sections were then prepared for immunohistochemistry by 10' postfixation in 4% paraformaldehyde in phosphate buffered saline (PBS) or unmasking of antigen sites by hydrating in PBS and then boiling in 0.01M sodium citrate and cooling on ice. This was followed by washes in PBS and deactivation of endogenous peroxidases by treatment with 0.5% hydrogen peroxide in 95% ethanol for 30', washes in PBS and PBS-Tween (0.05%). Non-immunological binding of sera was minimized by blocking for 90' in 20% newborn calf serum in 0.05% PBS-Tween.

Primary antibodies were applied from 90' at room temperature to overnight at 4°C, depending on the antibody (Table 1.).

Primary antibody incubation was followed by washes with PBS-Tween, and 90' incubation with secondary antibodies (anti-rabbit, 1:200, Vector) at room temperature. Secondary-antibody treated sections were washed and incubated with avidin-biotin complex 90' at room temperature, and then washed. Peroxidase was visualized histochemically by diaminobenzidine (DAB) reaction, which was carried out on wild-type and experimental sections for the same time-periods. Sections were then washed in water, dried and mounted with Aquatex (MERK). Stained sections were photographed with a digital AxioCam (Zeiss) camera.

Primary Antibody	concentration	incubation	unmasking	ABC	Host	Source
Calretinin	1:3000	O/N 4°C	no	yes	rabbit	Swant
Phospho histone H3	1:100	O/N 4°C	yes	yes	rabbit	Upstate
Tbr1	1:2500	O/N 4°C	yes	yes	rabbit	R. Hevner
Tbr2	1:700	O/N 4°C	yes	yes	rabbit	R. Hevner

Table 1. Primary antibodies and their dilutions.

Immunofluorescence

Immunofluorescence was carried out for COUP-TFI. Sections were washed in PBS and non-immunological serum binding was inhibited by blocking in a solution of 6% bovine serum albumin, 5% goat serum, 20mM MgCl2, 0.3% Tween in PBS. Sections were then incubated with primary antibody (Tripodi et al., 2004) for 90' diluted at 1:500 in blocking solution. After incubation, sections were washed with PBS and incubated with Alexafluor 568 conjugated- anti-rabbit secondary antibody (Alexa) for 60'. Sections were washed and mounted with Vectashield mounting medium (Vector) and photographed with a digital AxioCam (Zeiss) camera.

BrdU Staining

BrdU staining was carried out by injecting gravid dams with 100µg/g BrdU intraperitoneally and sacrificing the dam at the timepoint of interest. Embryos were dissected in PBS and fixed in 4% PFA overnight, passed through a sucrose gradient and embedded in OCT and sectioned.

Slides were then washed for 5' in PBS, followed by posfixation in 4%PFA and further washes. DNA was denatured by treating tissue in 2N HCl, 0,5% Triton X-100 at 37°C for 30 min and the tissue then neutralized by 0,1M Sodium Tetraborate buffer for 3x10 min at RT. The sections were then blocked in PBS; 10 % FCS; 0.1% Triton X-100 for 20 min at RT and incubated with the primary antibody diluted in blocking buffer overnight at 4°C (Sigma, anti-BrdU 1:300). The next day, sections were washed 3x8 min in the blocking buffer at RT and incubated with the secondary antibody diluted in blocking buffer 2 h at RT. Slides were then washed and mounted.

In Situ Hybridization

In situ hybridization was carried out on cryosectioned tissue using digoxigenin labelled probes. Probes were prepared by restriction endonuclease linearization of 5μg maxiprep DNA plasmid samples carrying the probe sequence. Digested and undigested samples were run on an agarose test-gel to check for linearization. 1μg of linearized and spin column-purified samples were then used for both sense and antisense transcription reactions by incubating 2μl DigMix 10x (digoxigenin label, Roche), 2μl transcription buffer 10x, 0.5μl RNase inhibitor, 2μl RNA polymerase (T3 or T7, Roche), 1μg linearized DNA and H_2O milliQ to volume of 20μl, at 37°C for 2 hours. After running a test gel for the presence of RNA, 2μl DNAse were added to each sample and incubated for 15 minutes at 37°C, after which DNAse action was quenched with 0.2μl EDTA 0.5M. Probes were precipitated by addition of 10μl LiCl 4M, 300μl EtOH 100% and 100μl H_2O milliQ and incubated overnight at -20°C. The following morning, precipitated probes were centrifuged (15', 13krpm at 4°C), the

supernatant removed and the pellet air-dried and then resuspended in 40μl H_2O milliQ.

Slides were then dried, washed, postfixed, treated with triethanolamine buffer and washed again with PBS. Sections were then incubated with prehybridisation solution prewarmed to 70°C for 1hour. Digoxigenin labelled probes prepared as described above were suspended in hybridisation buffer at a concentration of 300ng/ml and applied to sections. Slides were incubated overnight in a chamber humidified with a formamide-SSC solution at 70°C.

After incubation with the probe, sections were washed twice in one hour at 70°C in posthybridisation solution. Sections were then washed in a NaCl- maleic acid- tween solution (MABT) and prepared for immunological detection by blocking with MABT-sheep serum. Anti-digoxigenin antibody was then applied at a concentration of 1:2000 in blocking solution and incubated overnight at 4°C.

Slides were washed with MABT and a Tris-MgCl2-NaCl-Tween buffer, before antibody-binding was visualized by incubation with NBT-BCIP at room temperature. The color reaction was allowed to be carried out until a signal could be detected. The reaction was stopped in PBS-Tween, slides washed, dried and mounted in Aquatex (Merk). Sections were photographed with a digital AxioCam (Zeiss) camera.

Axonal tracing by DiI and DiA

Axons were labelled and traced by lipophilic dyes 1,1-dioctadecyl 3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes) or 4-[4-(dihexadecylamino)styryl]N-methyl-pyridinium iodide (DiA, Molecular Probes). Brains from E15.5 embryos were fixed at least overnight in 4% PFA in PBS, after which single crystals of DiI were placed in the prospective dorsal thalamus to label

thalamocortical axons and single crystals of DiA were placed in somatosensory cortex to label corticofugal axons. DiI and DiA were allowed to diffuse along axon tracts for 6 weeks while brains were stored in 4%PFA in PBS in the dark at 4°C. Brains were then embedded in 5% low-melting agarose and cut into 100μm coronal sections on a vibratome. The sections were mounted with vectashield with DAPI (Vector), and digital images were taken using an AxioCam (Zeiss) camera on a fluorescent microscope and processed in Adobe Photoshop.

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